THE CHEMICAL AND PHYSIOLOGICAL PROPERTIES OF THE INTERNAL SECRETIONS

E. C. DODDS AND F. DICKENS

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PREFACE

This volume has no claim to originality other than that it contains, under one cover, a summary of the literature dealing with the chemical aspects of the internal secretions. The descriptions are based upon a series of lectures delivered by one of us at the Middlesex Hospital. In the course of preparation we were impressed by the vast amount of references to be consulted, and by the absence of any collected account of the original papers. On the completion of this task we were tempted to publish our findings, mainly with the view to saving other workers the somewhat dreary task of looking up all the original researches.

All the references contained in this work have been consulted by ourselves or our colleagues, and have not been merely copied from other publications. The object has been, in all cases, to provide workable descriptions for the preparation and standardisation of the products. In some cases the former are dealt with in rather a nebulous manner, this being due to the fact that only patent literature was available.

In order to render the book of general interest, a summary of the physiological actions has been given in each case. In some instances this is rather condensed, owing to the impossibility of entering into the realms of pure physiology in a book of this size. For similar reasons, the clinical applications of insulin are only referred to briefly, since a treatise on the treatment of diabetes mellitus would be out of place here.

Although we have endeavoured to eliminate personal

views, we have indicated that certain points are controversial, and in some cases unconfirmed.

The criticism may be offered that too much unconfirmed work has been included. Our answer to that is twofold: in the first place, any worker investigating the properties of a new product would have, of necessity, to make himself acquainted with the previous researches on the subject; and secondly, recent advances have demonstrated the folly of over-scepticism. In support of this may be quoted the comparatively obscure researches of Zuelzer on insulin, and that of Fränkel and Herrmann upon the ovarian hormone.

In conclusion, it is a pleasure to thank Miss Agnew and Mr. J. D. Carruthers (of the Winter Distress League) for their valuable help in our search through the literature. We have also to thank the publishers for their courtesy and help in the course of preparation of the book.

E. C. D.
F. D.

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INTRODUCTION

The origin of theories concerning the internal secretions dates back many hundreds of years, although these early views were completely without experimental support. The theories were, for the most part, wild and speculative, and it was not until the nineteenth century that these problems were attacked in a logical way. The latter half of the last century saw almost all the pioneer work upon the organs of internal secretion. Thus Addison focussed the attention of physiologists and chemists upon the suprarenal glands ; Marie provided the stimulus for work upon the pituitary body, whilst Kocher and von Mering and Minkowski were responsible for the earlier investigations upon the thyroid and pancreas. Other workers, such as Brown-Séquard, demonstrated the endocrine functions of the reproductive organs. This pioneer work was rounded off by the researches of Schafer and his colleagues upon the effects of extracts of these glands when injected subcutaneously or intravenously. These investigations have become the foundation of modern therapy in this field.

By the year 1900 the physiological importance of practically all the organs of internal secretion had been fully established, and it remained for workers to improve the methods of preparation and to inquire into the chemical composition of the principles. The first achievement was the discovery of the constitution of adrenaline, and this was quickly followed by Stolz's synthesis in 1904. After several years' work, Kendall isolated the active principle of the thyroid, and stated, in 1917, that a small quantity of thyroxin had been synthetised.

One of the most notable advances is the isolation of insulin by Banting and Best, who have provided both the clinician and the biochemist with a substance of great practical and theoretical interest.

Although it is as yet early to offer any opinion, it would appear that the recent work upon the ovarian hormone will prove to be of the very greatest importance. Quite apart from its clinical value, which is bound to be very great, the substance opens up entirely new fields of research. Here we have a body the injection of a few milligrams of which causes, within forty-eight hours, a solid column of cells to become a tube, in addition to the other cellular changes occurring in œstrus. That the subcutaneous injection of this hormone can influence so powerfully the growth of a series of epithelial cells is surely something entirely new. It is justifiable to say that hitherto no body has been isolated with such a powerful action upon the growth of cells, and it will not be surprising if this and similar bodies start a new era in the investigation of cell growth, and possibly of malignant disease.

Another property of internal secretions is their amazing potency. Thus dilutions of one in twenty millions of pituitary extracts will cause contraction of plain muscle, and less than a tenth of a milligram of the purest insulin obtained will produce hypoglycæmia in a two-kilogram rabbit. These properties alone add a great deal of chemical and physiological interest to the products of the endocrine organs.

In the following pages will be found a description of the history, methods of preparation, and chemical and physiological properties of the various internal secretions. In so far as is possible, the inter-relations of these bodies have been emphasised, but the accounts have, of necessity, to be considered under the heading of each substance. It must be realised that in the body the end result is due to the mean, as it were, of the actions of all the internal secretions—a fact rather lost sight of in dealing with the effect of administration of any one principle.

CHAPTER I

THE INTERNAL SECRETION OF THE PANCREAS (INSULIN)

Historical

THE history concerning the scientific study of pancreatic function may be said to have originated in the researches of Brunner (1) towards the end of the seventeenth century. Diabetes was, at that time, unrecognised as a clinical entity, and although there is little doubt that Conrad Brunner produced, by the extirpation of the pancreas, symptoms identical with those of diabetes, many years elapsed before the problem was taken up again by later workers. The post-mortem examination of persons dying from diabetes had revealed changes in the pancreas which led experimentalists to investigate the question from the point where Brunner had left it. Little success attended these experiments until von Mering and Minkowski (2) in 1885 succeeded in producing experimental diabetes in animals by removal of the pancreas, and in the series of researches following their first experiments they were able to elucidate the problem of diabetes mellitus so completely that Minkowski was led to postulate the removal from the organism of some active principle essential for carbohydrate metabolism. Thus at this early date Minkowski regarded the pancreas as an organ of internal secretion, although the suggestion was not definitely made until later, when Lépine (3) used the term in this connection.

Meanwhile the study of the microscopical anatomy of the pancreas was receiving attention. In 1869 Langerhans described the islets and recognised them as distinct from the acinar tissue which is responsible for the production

of the digestive secretion of the pancreas, whilst other workers, notably Laguesse (4) and Diamare (5), had been led to believe that the islet tissue is concerned in the production of an internal secretion whose function it is to control the metabolism of carbohydrates. This hypothesis was by no means proved at the time. The view was considerably strengthened, however, when Minkowski (6) and also Hédon (7) showed that a portion of the pancreas transplanted under the skin prevents the occurrence of diabetes when the main gland is removed. Opie (8) and others have described definite changes in the histology of the islet cells in the pancreas of patients dying from diabetes: thus very strong support is given to the view that the islets play some definite part in the production of the internal secretion. Nevertheless, the existence of this secretion could only be definitely shown when active extracts had been isolated from the pancreas and had been used to affect the metabolism of sugar in another animal. A number of workers had already attacked this problem, mostly without success, with the result that many denied the existence of the hormone. Others, however, took the correct view, namely, that the active principle is destroyed by the action of enzymes in the extract, or by some defect in the method of extraction. It is unnecessary to consider these numerous unsuccessful attempts in any detail. That this extraction would ultimately prove successful was believed by many workers, and Schafer (9) as early as 1916 suggested the name insulin for the hormone, indicating the supposed source of the hormone in the islets of Langerhans.

Zuelzer's Preparation.—The most interesting observations on the extraction of insulin at about this time were those of Zuelzer (10), who came very near to solving the problem in 1908. He prepared both aqueous and alcoholic extracts of the pancreas, and there is no doubt that he obtained an active substance. One of his methods of preparation was as follows: The minced pancreas was allowed to stand with sufficient sodium bicarbonate to produce a weakly alkaline reaction. The liquid was

pressed out, and alcohol added to precipitate the albumin. The filtered solution was then concentrated in a vacuum-still, and the final product was obtained as a fine, dry, grey powder. When this process is compared with those used by modern workers, the similarity becomes very striking. Zuelzer administered the extract to diabetics, with successful results in some cases. Other workers, however, were led to abandon the treatment on account of the toxic effect of the substance. Zuelzer states that his extract gave none of the known protein tests, but it is difficult to see how an extract prepared by his method could be entirely free from protein, and at the same time contain any active principle. Zuelzer was aware of the action of his extract in reducing the hyperglycæmia and glycosuria produced by adrenaline, and used this as a method of standardising the product. In common with other earlier workers he was greatly handicapped by the lack of suitable methods for the estimation of blood-sugar, and it is possible that the symptoms which he regarded as indications of toxicity were in some cases due to hypoglycæmia.

Rennie and Fraser's Observations (*Fish Islets*).—Rennie and Fraser (11) took advantage of the fact that certain teleostean fishes have a very large, distinct islet, which can be dissected out. From a number of these it is easy to prepare an extract of islet tissue in normal saline. In the majority of cases in which these extracts were administered to diabetic patients, they were given orally, so that it is not surprising, in the light of subsequent knowledge, that no beneficial results were observed. In one case subcutaneous injections were given, but the extract produced very marked toxic effects.

Knowlton and Starling's Method.—Knowlton and Starling (12) performed some very important researches upon the nature of the pancreatic hormone. After evolving their technique for the surviving heart-lung preparation, they realised that it might be applied to the problem of diabetes. Knowlton and Starling found that sugar disappeared from the perfusing blood at the rate of about

4 mg. per gram of heart muscle per minute. If, however, the metabolism of a preparation derived from a depancreatised dog were investigated, the rate of disappearance was greatly reduced. They therefore concluded that such a preparation would be suitable for testing the potency of pancreatic extracts. In the light of recent developments, the following paragraph from their paper, written early in 1912, is particularly striking: "Reasoning from the behaviour of substances belonging to the class of hormones, such as secretin and adrenaline, we might guess that the pancreatic hormone would be a body diffusible, soluble in water, unstable in alkaline solution but more stable in slightly acid solution, and not destroyed immediately at the temperature of boiling water". The accuracy of this deduction can be easily appreciated on reference to the section on the properties of insulin. Knowlton and Starling therefore ground up fresh pancreas with sand and N/10 hydrochloric acid in sufficient quantity to keep the reaction fairly acid. The paste was extracted with acidified Ringer's solution, and the mixture was brought rapidly to the boil. The reaction was maintained slightly on the acid side throughout the heating, more hydrochloric acid being added if necessary. The mixture was filtered and neutralised. It can be seen that Knowlton and Starling adopted a method which destroyed the tryptic activity. They found that addition of this extract to a diabetic heart-lung preparation restored the normal rate of sugar disappearance, and they justifiably concluded that they had demonstrated the presence of an antidiabetic hormone. Their paper is also of the greatest importance in that it proves conclusively that the results of pancreatectomy can be explained by the absence of an internal secretion, antidiabetic in character, of the gland.

Murlin and Kramer's Alkaline Extract.—Murlin and Kramer (13) prepared alkaline extracts of the pancreas, the administration of which reduced the glycosuria of depancreatised dogs. The administration of alkali alone, however, was found to give the same effect.

Kleiner's Observations on the Effect of Extracts upon the Blood-sugar.—Kleiner (14) was one of the first investigators to control his results by blood-sugar investigations. He found that the administration of watery extracts of fresh dogs' pancreas produced a marked decrease in the blood-sugar and a corresponding decrease in the urinary sugar. These experiments were confirmed later by Banting and Best (15), who found, however, that anuria was also produced in the experimental animals by the use of an extract prepared by this method.

Schulze (16) and Ssobolew (17) had found independently that degeneration of the acinar tissue accompanies blockage of the secreting ducts, but that the islets are not affected under these conditions.

Scott's Alcoholic Extract.—E. L. Scott (18) repeated and confirmed these experiments and, in 1911, was one of the first to use alcohol for the purpose of inhibiting the actions of the external secretion. Unfortunately he used alcohol of such concentration that the greater part of the insulin must have remained unextracted.

Scott's method was as follows: The minced glands were extracted with alcohol and the alcoholic extract concentrated *in vacuo*. Fat was removed from the residue by extraction with ether, and the ether extract discarded. The residue was dissolved in 95 per cent. alcohol, in which insulin is only sparingly soluble, so that much insulin must have been rejected at this stage. This work of Scott's is of great interest in the light of the subsequent observations of Banting and Best, which will now be described.

The Toronto Workers

In 1921 work was begun at the Department of Physiology of the University of Toronto by Banting and Best which led to the isolation of the active principle of the internal secretion of the pancreas in a state sufficiently pure for administration to diabetic patients. The hypothesis upon which these experiments were based was that

some constituent of the external secretion of the pancreas was antagonistic to the internal secretion. This hypothesis was suggested to Macleod, and on his advice the experiments were carried out. If the above hypothesis be correct, the inhibition of the action of the external secretion would be necessary if a good yield of insulin were to be obtained. In the first experiments this problem was attacked in the following way. The pancreatic ducts of a number of normal dogs were ligated, thus bringing about the degeneration of the acinar tissue and cutting off the supply of the external secretion, as is mentioned above. The animals recovered quickly from the operation, and showed no signs or symptoms of diabetes. Ten weeks later, in July 1921, Banting and Best killed the dogs by chloroform, and prepared extracts by grinding the excised gland in a mortar with sand and ice-cold Ringer's solution. The liquid was filtered through paper, and the filtrate was administered intravenously to diabetic dogs. A prompt and decided lowering of the blood-sugar, accompanied by a fall in the urinary sugar, resulted. The sugar tolerance was also increased. These actions on the blood-sugar were destroyed by incubation of the extract with trypsin in alkaline solution, whereas in the absence of trypsin in faintly acid solution the activity remained. Extracts of spleen and liver similarly prepared had no effect, neither had active pancreatic extracts when administered by the mouth or by the rectum. These results have been amply confirmed since, and to Banting and Best, therefore, must be given the credit of first obtaining an extract which could be consistently prepared in an active state. It is evident, however, that the method is not a practical one for obtaining insulin in any quantity, and for this reason these investigators turned their attention to other supplies of pancreas which were available. Ibrahim (19) had established the fact that active proteolytic enzymes are not present in the pancreas of the fœtal calf up to the fourth month. Banting and Best (20) took advantage of this fact, and were consequently able to prepare extracts containing comparatively large quantities

of the substance. They also prepared active extracts from the normal pancreas of the ox by alcoholic extraction, thus following the work of Zuelzer and of Scott.

Alcohol Extraction Processes

These earlier extracts were prepared by the extraction of finely divided ox-pancreas with alcohol containing 0·2 per cent. of hydrochloric acid. In this, and all the methods subsequently described, it is essential that the pancreas should be extracted not more than about two hours after the death of the animal, if satisfactory yields are to be obtained. If this is not the case, tryptic action will destroy the insulin. The concentration of alcohol in the mixture was in some cases as high as 60 per cent. The liquid was removed from the glands by filtration and the filtrate was evaporated at a low temperature. By this means an aqueous residue was obtained containing the active principle, together with some fats and protein; about 5 or 10 c.c. contained one dose suitable for a depancreatised dog. It was shown that a completely depancreatised dog could be kept alive for at least ten weeks by administration of this crude extract. It was thus shown that the active principle could be obtained from a source so readily available as ox-pancreas. These extracts, however, were not sufficiently pure for satisfactory administration to diabetic patients, either because of local irritation produced on injection or because of the general toxic properties.

The purification of the crude extracts was accomplished by Collip (21), who joined the original workers in December 1921. Collip's method of purification depends on the fact that insulin is soluble in alcohol of 80 per cent. concentration or less, but in more concentrated alcohol—over 90 per cent.—it is insoluble. The latter fact had already been established by Banting and Best, who found that none of the insulin contained in the dried residue obtained by the complete evaporation of their extract was soluble in 95 per cent. alcohol. They did not, however, apply

this method to the purification of crude insulin before the work referred to above. This method consists essentially of a fractional precipitation of the extract by means of increasing concentrations of alcohol. It is briefly as follows: 95 per cent. alcohol is added to freshly minced pancreas until a volume of alcohol equal to the weight of pancreas taken has been used. The mixture is stirred at intervals during a few hours, after which the liquid is filtered off. Alcohol is then added to the filtrate, to bring the concentration of alcohol in the latter (which is now only about 60 per cent. owing to the water in the pancreas) up to 80 per cent. The precipitate of inactive proteins thus formed is filtered off. The filtrate is then concentrated to small volume by distillation *in vacuo*. Extraction with ether removes lipoids and fat, and the watery solution is then concentrated to a pasty consistency. This material is extracted by means of 80 per cent. alcohol, in which the active principle is entirely soluble. The addition of several volumes of 95 per cent. alcohol completely precipitates the insulin in such a state that it may be collected on a Büchner funnel, and then dissolved in water for clinical use. The clinical effects of this solution are reported in a paper by Banting, Best, Collip, Campbell and Fletcher (22). This method of extraction was found to be fairly satisfactory at first on a small scale, but was unsuitable for large-scale work, since consistent results were not obtained.

It was not long, therefore, before modifications were suggested. The first of these to be applied to the commercial production of insulin (by the Eli Lilly Company) is of interest. It is very similar to the method described above, except in the use of acetone acidified with formic or acetic acid (not more than 0·1 per cent.) for the preliminary extraction of the pancreas. After allowing the mixture of pancreas and this acid-acetone, in equal proportions, to stand for several hours, the liquid is pressed out, filtered and evaporated. This was accomplished by transferring the filtrate to shallow trays, which were placed in a tunnel through which a current of hot air was rapidly

drawn. The liquid was evaporated to one-tenth of its volume in about one hour, the temperature never exceeding 35° C. The residue was chilled, and the lipoid material which separated was filtered off. From this stage a method practically identical with Collip's purification process, described above, was followed. This process gave consistent results, but the yield obtained was still very low.

Benzoic Acid Method.—Insulin is readily adsorbed by many substances, and is carried down with them on precipitation. This fact was made the basis of a process of preparation by Moloney and Findlay (23). The method is briefly as follows: the minced glands are extracted with alcohol and the filtrate concentrated by distillation *in vacuo*. To each litre of the concentrated aqueous solution 50 c.c. of a 25 per cent. solution of sodium benzoate and 12·5 c.c. of concentrated hydrochloric acid are added. By this means a saturated solution of benzoic acid in the concentrated liquor is obtained. In order to produce a precipitate of benzoic acid, a further 40 c.c. of 25 per cent. sodium benzoate and 10 c.c. of hydrochloric acid are then added. The precipitate carries down with it about two-thirds of the active material. A further quantity is obtained by repeating the precipitation, using another 40 c.c. of 25 per cent. benzoic acid solution and 10 c.c. of hydrochloric acid. The benzoic acid precipitates are mixed and dissolved in a small volume of 80 per cent. alcohol, the insoluble portion being rejected. The solution is then concentrated to dryness *in vacuo* and the benzoic acid is removed from the residue by extraction with ether, and the insulin is dissolved in a small volume of water.

The use of the benzoic acid method gave a product which contained less protein than that obtained previously, and the toxic effects of insulin obtained by the method were much less than those of the earlier extracts. The great practical objection to the method lies in the difficulty of separating the benzoic acid precipitates, the filtrations of which are long and tedious. This objection applied

also to the preliminary filtration of the alcohol extract of the minced glands in this and other processes depending upon alcoholic extraction.

The Ammonium Sulphate Method.—In the method of Doisy, Somogyi and Shaffer (24) an alcoholic extract of the glands is made in the same way as in the preceding section, and the alcohol is distilled off *in vacuo*. The aqueous residue is then half saturated with ammonium sulphate, when a precipitate appears which contains almost all the active material.

One kg. of finely ground pancreas is stirred with 40 c.c. of 10 N sulphuric acid (or 40 c.c. concentrated hydrochloric acid), 1200 c.c. of 95 per cent. alcohol and 300 c.c. of water. The mixture is stirred well at intervals during four to twelve hours at room temperature, when it is strained through cloth and pressed out. The solid is re-extracted with a litre or more of 60 per cent. alcohol, and is again strained and pressed. The combined extracts are made only faintly acid to litmus by addition of sodium hydroxide, and are then filtered through paper. The filtrate is evaporated at 20-30° C. by exposing it in shallow trays to a draught of warm air, until all the alcohol is removed. The liquid is then transferred to a separating funnel, acidified slightly, and 40 g. of solid ammonium sulphate are added for each 100 c.c.: the mixture is shaken until all is dissolved. The precipitate which rises to the surface is separated, drained and dissolved in 75 per cent. alcohol. The insoluble matter is removed in the centrifuge and the clear liquid is pipetted off. To it are added 8 to 10 volumes of 95 per cent. alcohol. The reaction is adjusted to pH 5 to 6, and the precipitate of insulin collected after some hours. For clinical use it needs to be still further purified.

Fenger and Wilson (25), who used the process, emphasise the need for absolutely fresh pancreas, and state that mincing alone is not quite sufficient, but that a subsequent grinding process assists in breaking the cell walls, and thus making the insulin more accessible.

Fisher's method (26) is a modification of the process of

Doisy, Somogyi and Shaffer. Great stress is laid on the separation of the toxic and antidiabetic fractions from the crude product. The method is as follows: the ox-pancreas is conveyed to the laboratory, cut into small pieces and immersed in the acid-water-alcohol mixture of Doisy, Somogyi and Shaffer (see above). After mincing, the tissue was frozen at -10° C. and thawed again, with a view to rupturing the cells and liberating the insulin. This procedure has been adversely criticised by Fenger and Wilson. The same liquid was now once more added to the pancreas, and the Doisy, Somogyi and Shaffer method followed from this stage. In the final precipitation, when 9 volumes of alcohol were added to the liquid which had been centrifuged, Fisher noticed that two precipitates were thrown down. Fractional precipitation was resorted to in order to separate the two precipitates. Addition of 1 volume of alcohol produced a dark grey precipitate. This was filtered off, and a further 8 volumes of alcohol were added. The second precipitate produced was pure white.

Fisher showed conclusively that the first precipitate is toxic, and when injected caused sterile abscesses at the site of injection. This fraction raised the blood-sugar, and caused the death of rats and guinea-pigs by excessive stimulation of the medullary centres. The other precipitate is the active fraction (insulin), and is the specific antidiabetic hormone. The yield of insulin per kilogram of pancreas is proportional to the degree to which the toxic fraction is removed This work is very valuable in explaining the delayed action of certain insulin preparations, and will be referred to later.

The Alkaline Extraction Method.—This method is due to Dudley and Starling (32), and involves two distinct processes, the first of which is an extraction of the pancreas by means of alcohol made alkaline by the addition of sodium bicarbonate, and the second is a purification process. The details of the method are as follows: 5 kg. of ox-pancreas are minced through a sausage machine into 5 litres of 95 per cent. alcohol; 425 g. of

sodium bicarbonate are stirred into the mixture, which is then re-minced. The minced tissue is strained off on a fine sieve, and is re-minced into the alcohol once more. The mixture of alcohol and pancreas is allowed to stand at room temperature for two hours. It is then poured on to a stout cloth, transferred to a suitable press, and as much liquid as possible pressed out. The turbid liquid (about pH 7·5) is treated with 1½ times its volume of 95 per cent. alcohol, and is placed in the cold room at -3 C. overnight. It is then filtered through a folded paper, and 50 c.c. of glacial acetic acid are added to the filtrate which is evaporated *in vacuo* from a water-bath at 40-50 C. until the volume of the residue is about 750 c.c. The fat is then removed by shaking with light petroleum, and the aqueous layer treated with 4 volumes of absolute alcohol, to bring the concentration of alcohol in the mixture up to 80 per cent. A syrupy precipitate settles on leaving in the cold room overnight. The next morning the supernatant liquid is decanted, and the concentration of alcohol is made up to 93 per cent. by the addition of 2 volumes of absolute alcohol. A precipitate forms, and is allowed to settle from the liquid, which is kept in the cold room for fifteen to twenty hours for this purpose.

This precipitate is obtained as a white granular powder (about 10 g.), which is washed with absolute alcohol and dry ether in the centrifuge. This substance is crude insulin, and is very hygroscopic. It should therefore be placed in a vacuum-desiccator, and dried over sulphuric acid without delay. The purification of this product by Dudley's picrate method is dealt with later. This method of extraction gives good yields of insulin, but is apt to be troublesome practically, owing to the tedious filtrations. A very large volume of alcohol is also required for each 5 kg. of pancreas. For this reason acid extraction methods, though probably not giving quite such a good yield of insulin, are in more general use.

Acid Extraction Processes.—As an example of a method in which acid alcohol is used as the extracting fluid, the method of Best and Scott (27) will be described. In this

method many of the steps in the earlier processes already described have been incorporated.

Fresh glands are obtained at intervals of three hours from the slaughter-house, where they are dissected free from fat and connective tissue. The glands are weighed, and then finely minced. The minced material is transferred to jars containing an equal weight of 95 per cent. alcohol acidified with 1·3 per cent. of acetic acid. This mixture is slowly agitated for three hours, and at the end of this time it is poured into a rotary centrifuge which separates the alcoholic extract from the solid matter. The latter is re-extracted for three hours with a volume of alcohol (60 per cent.) equal to that removed after the first extraction. The first and second extracts are combined, made neutral to litmus with sodium hydroxide, and chilled to 0° C. The liquid is then filtered, and the clear alcoholic extract concentrated to about one-twentieth of its original volume in an efficient vacuum-still, the temperature not being allowed to rise above 30° C. After the distillation the concentrate is quickly heated to 55° C. The fatty mass which rises to the surface at this temperature is easily skimmed off. Fats and lipoids are removed from this portion by ether extraction, and the aqueous portion which settles from this ether extract contains about one-fourth of the total potency of the extract. It is made up to 80 per cent. alcohol by the addition of 95 per cent. spirit. This mixture is filtered through paper (A—see below).

Meanwhile ammonium sulphate is added to the main portion of the concentrate to secure half saturation (37 g. per 100 c.c.). This mixture is stirred well, and almost immediately protein material separates and rises to the top of the liquid. After standing for half an hour this is skimmed off and is drained on a hardened filter-paper for three to six hours. It is then added to sufficient 95 per cent. alcohol to ensure a final concentration of 75 to 80 per cent. alcohol. The solution is filtered from the precipitated protein and this filtrate is mixed with that obtained above (A). The active principle in these

combined filtrates is precipitated by adding to them an equal volume of ether. This precipitate settles overnight and the supernatant liquid is decanted.

The precipitate is dried *in vacuo* and is then treated with dilute aqueous ammonia of such concentration that the pH of the resulting solution is approximately 8. The insulin completely dissolves, and the pH of the solution is then adjusted to 3·5. At this hydrogen ion concentration a dark precipitate of impurity separates and is discarded. The filtrate, which is an aqueous extract containing the active principle in solution, may be purified by either of the methods described later.

The Aqueous Extraction of Insulin

The expense involved in the use of large volumes of alcohol or acetone has caused many workers to investigate aqueous methods of extraction. As has already been mentioned, many of the earlier investigators had employed aqueous extracts. Thus Zuelzer and, later, Knowlton and Starling employed methods which began with aqueous extraction, the former using alkaline and the latter workers acid media. Banting and Best, in their first experiments, prepared their extracts by grinding the pancreas with ice-cold Ringer's solution. In all these experiments the yields of insulin were probably very poor owing to the unrestricted action of proteolytic enzymes, and for this reason Banting and Best and subsequent workers turned their attention to alcoholic extraction. More recently, interest in the watery extraction of insulin has been reawakened, partly on economic grounds and partly on the ground that insulin is more soluble in watery than in alcoholic solvents. Since water forms the natural medium of the substance in the body, it is reasonable to suppose that a more efficient extraction should be possible by watery than by alcoholic solvents, if the tryptic action could be reduced to a minimum.

There are, however, many practical difficulties in the preparation of aqueous extracts of the pancreas, chief of

these being the difficulty attending the filtration of the minced glands from the aqueous extract. This is often exceedingly slow and tedious, and during this process the proteolytic enzymes in the extract have time to destroy a large part of the active principle.

Piper, Allen and Murlin's Method. — This objection applies to the methods of Piper, Allen and Murlin (28-29). In this preparation the ox-pancreas is thoroughly ground with 4 volumes of 0·2 N hydrochloric acid ; the mixture is brought to 75° C. and held at this temperature for one hour. After cooling and skimming off the fat, the mixture is filtered through cheese cloth, and neutralised with N sodium hydroxide to a pH of 4·9. It is then allowed to filter through a coarse filter-paper overnight. It is difficult to see how any appreciable quantity of active principle survives this treatment. The insulin, together with protein, is separated by the solution of 250 g. of sodium chloride per litre of filtrate. The precipitate is purified from a large part of the proteins by alcohol, and finally by an iso-electric precipitation.

Best and Scott's Method.—Best and Scott (*loc. cit.*) state that they have tried some 150 experiments, using different modifications in the method of extraction. The yields obtained by any of these methods, however, do not compare with that given by alcohol or acetone extraction. A typical experiment is given as follows : two pounds of minced pancreas were added to 1500 c.c. of distilled water acidified with 3·5 c.c. of concentrated sulphuric acid. The mixture, after extraction for two hours in the cold, was filtered through paper. Best and Scott state that the mixture filters readily if the pH of the filtrate is approximately 3·5. The exact details of the methods used for the separation of the insulin in the filtrate are not given, but the yield obtained by this method under the most favourable conditions was about one-quarter of that obtained by the same authors' alcohol method.

Acetone Picrate Method.—The present writers (30) have worked out an aqueous method of extraction which gives yields of active material comparable with those obtained

by the various alcohol processes. The method is based upon some preliminary experiments (31) upon the properties of a series of picrates, prepared by the addition of picric acid solution to aqueous solutions of a series of typical proteins. The differential action of various solvents upon these picrates and upon the picrate prepared from solutions containing purified insulin was investigated. In the majority of solvents there was little difference in the behaviour of insulin picrate and of the other picrates investigated. The most striking differences were found in the action of acetone. Insulin picrate is very readily soluble in aqueous acetone, whereas the other picrates investigated were almost insoluble. The optimum concentration of acetone is about 70 per cent., for it was found that the picrate of insulin dissolves most readily in aqueous acetone of about this concentration.

These observations at once showed how the insulin, in the form of its picrate, could be quickly separated from the mass of protein associated with insulin in any aqueous extract. This step had been the difficulty in previous methods of aqueous extraction. Outlined briefly, the original process consisted in the extraction of insulin and protein from the pancreas by chilled aqueous formic acid, and precipitation of the protein and insulin completely from the fluid by the addition of saturated aqueous picric acid. The precipitate was filtered off and the moist picrates extracted by means of acetone, thus dissolving out the insulin picrate whilst the bulk of the protein picrate is left undissolved.

The insulin can be prepared from this picrate, as Dudley (32) had shown by dissolving it in alcohol containing hydrochloric acid, which converts the picrate into the hydrochloride. The latter is precipitated, on the addition of acetone, as a white powder, which consists of fairly pure insulin hydrochloride, apparently differing in no way from that obtained by the alcohol processes.

This method, besides effecting a great economy in alcohol, substantially reduces the time taken in the preparation. The yields were comparable with those

obtained by alcoholic extraction, and could be consistently repeated. Nevertheless, the great objection which applies equally to this and other methods of aqueous extraction had not been obviated, namely, the first tedious filtration of the minced glands. It was evident that even in the acid fluid used for extraction, tryptic action must have caused considerable loss of activity, owing to the time taken.

In order to minimise this action, several substances which are known to inhibit the action of trypsin were added to the extracting liquid. The only one that was found to be of use for this purpose was paraldehyde. The yield obtained was doubled by this means, and by the use of other improvements, but the preliminary filtration was troublesome on a large scale owing to the gelatinous nature of the material.

In view of the ready solubility of insulin picrate in 70 per cent. acetone and the insolubility of protein picrates, it was an important point to ascertain whether this preliminary aqueous extraction of the pancreas was necessary, and whether picrates could not be prepared simply by grinding pancreas with solid picric acid. The application of picric acid to the original tissue, as a step in the extraction of insulin, was discussed in the course of a conversation with Dr. Dudley, who informed us that it was already under trial by him for fish islets.

One kg. of pancreas was minced and well mixed with 45·0 g. of dry, powdered picric acid. After thorough mixing the whole was re-minced twice. A uniform, pasty, yellow mass was formed. This was extracted with 500 c.c. of dry acetone, and the clear liquid was separated by filtration and by pressure through "jean". The second and third extracts were made with 70 per cent. acetone. The separation of liquid is very complete, and the solid material in the press should be quite dry. The acetone-picrate solution was then freed from acetone by distillation *in vacuo*, and the precipitation of the picrate rendered complete by the addition of an equal volume of saturated picric acid. The precipitate was collected and stirred

with dry ether, whereby the fat and excess of picric acid were removed, leaving the pale yellow amorphous precipitate of picrate, which is then converted into the hydrochloride in the manner to be described later. The average yield of hydrochloride by this method was equivalent to about 1000 rabbit units from 1 kg. of pancreas, with a rabbit unit of 0·5 to 1 mg.

Some batches have shown even better results, the rabbit unit being as low as 0·25 mg. for the unpurified precipitate. In one experiment we have obtained from pig-pancreas a yield of 4280 rabbit units from 1 kg. of pancreas by this method.

After a series of experiments the following was adopted as the final method: the pancreas is minced and well stirred with finely powdered picric acid (45 g. per kg. of pancreas), which has been previously drained on a Büchner funnel. The mixture is again passed through the mincer to ensure even mixing. Water usually separates during the mincing, and is pressed out in a hand-press through "jean" without difficulty. From the well-mixed, picrated mass the picrate is then extracted with acetone. Three extractions are necessary, and the concentration of acetone in the extracting fluid should be approximately 70 per cent. The quantity of acetone used in the first extract will vary with the amount of water present in the picric acid-pancreas mixture. We have obtained the best results by employing absolute acetone for the first extraction in sufficient quantity to make the final concentration up to 70 per cent. In the succeeding extractions aqueous acetone containing 70 per cent. of acetone should be used, in quantity equal to half the weight of pancreas taken.

The method of extraction is as follows: the minced picric acid-pancreas mixture is stirred with the requisite quantity of acetone, and efficient mixing is ensured by passing the whole mass through the mincer once again. This mixing should produce a paste of a creamy consistency, from which the liquid extract is pressed through a double layer of "jean". When no more liquid can be pressed out, the solid material remaining is again minced

into the required volume of 70 per cent. acetone, and is re-extracted as before. This process is repeated once more.

The combined extracts are filtered if necessary, and the filtrate distilled *in vacuo* at about 50° C. until all the acetone is removed. From the aqueous residue, after cooling, a deposit of the amorphous precipitate of the picrate, together with some fat and crystals of picric acid, separates. It is as well to add an equal volume of saturated aqueous picric acid in order to ensure complete precipitation. If the precipitate contains a large amount of picric acid crystals the addition of the saturated picric acid solution is of course superfluous. The precipitate is collected on a Büchner funnel, washed by stirring with ether, and the ether is filtered off. The picrate remains undissolved, whilst the fat and excess of picric acid are removed. So obtained, the picrate is a pale yellow, amorphous powder, which is readily converted into the hydrochloride by Dudley's method (32).

For this purpose it is dissolved in acid alcohol prepared by mixing 25 c.c. of aqueous 3 N hydrochloric acid with 75 c.c. of alcohol. Ten to twenty c.c. of this mixture are usually required for each gram of picrate. By careful rubbing with a glass rod, a turbid solution of a dark brown colour is obtained. The solution is then centrifugated, and the supernatant fluid is poured off. The solid material in the bottom of the tube is ground up with a further quantity of the acid alcohol solution, and is centrifugated again. It is as well, though not absolutely necessary, to extract a third time. From the resulting clear fluid the hydrochloride is precipitated by the addition of 10 to 20 volumes of acetone. The hydrochloride is allowed to settle, and the clear supernatant fluid is decanted. The remainder is poured on to a Büchner funnel, and the precipitate is washed with acetone until free from picric acid, and finally with dry ether. It is then dried in a vacuum desiccator. The crude hydrochloride so obtained is a perfectly white, non-hygroscopic, amorphous powder, the rabbit unit of which lies between 0·25 and 1 mg.

The Purification of Crude Insulin

The insulin obtained by almost all processes of extraction requires to be purified before it is fit for clinical use. As will be described later, the method of physiological assay of insulin indicates the weight of substance containing one "rabbit unit", and this should not exceed approximately 0·5 mg. The impurities present in crude insulin consist of proteins which have been incompletely removed during the extraction process, and injection of a solution containing larger quantities of protein than that mentioned above is liable to cause local irritation and the formation of sterile abscesses at the place of injection.

The purification is effected in a variety of ways. The use of alcohol, as in the method of Collip (21), produces a substance the rabbit unit of which is approximately 5-10 mg. The insulin prepared by this method is therefore insufficiently protein-free for immediate clinical use, and may be purified by any of the methods described below. The same applies to crude insulin prepared by other methods.

The principal methods in use are: (1) the iso-electric precipitation method, (2) adsorption methods, (3) Dudley's picrate method.

1. *Iso-electric Precipitation.*—This method depends on the fact, observed by Doisy, Somogyi and Shaffer (24), and, independently, by Walden working in the laboratories of the Eli Lilly Company, that aqueous solutions containing insulin precipitate part of the active principle when the hydrogen ion concentration of the solution is adjusted to a certain definite value. This precipitate contains the insulin in a very active form. For the purification (33) the crude insulin is dissolved in water with the addition of just enough 0·1 N ammonium hydroxide to make the reaction just yellow to methyl red (pH 6 to 8). The insulin dissolves, and is separated from insoluble protein by centrifugation. The solution and washings of the precipitate are diluted to about 100 c.c. for each kg. of pancreas used. On adding dilute acetic

acid to about pH 5 a flocculent precipitate forms, which is centrifugated, and dissolved in a slight excess of 0·1 N hydrochloric acid.

From the mother liquor more active precipitate usually forms on standing in an ice-box for a few days, and this is added to the main fraction.

The solution may be pure enough for clinical use, but it is desirable to repurify it as follows : the precipitate at pH 5 mentioned above, after washing in the centrifuge with water at pH 5, is dissolved in a measured volume of N/10 acetic acid (5 or 10 c.c. for each kg. of pancreas represented). Exactly 20 per cent. of the equivalent quantity of sodium hydroxide solution is then added. This mixture produces a buffer solution of very closely pH 4, in which the insulin is soluble, but from which the " acid protein " is mostly precipitated. After standing in the cold room for some hours this is centrifugated, and the precipitate is washed. The washings are added to the original solution, and an amount of sodium hydroxide solution equivalent to exactly half the acetic acid used is added. This gives a solution of pH 5, in which the insulin is insoluble. It is collected by centrifugating, and is washed once or twice with distilled water. It dissolves readily in water to which have been added a few drops of dilute hydrochloric acid. The insulin obtained by this process is exceptionally pure.

According to some observations of Best and Scott (27) with insulin solutions containing an added protein, the precipitate formed at the iso-electric point of that protein may contain all the activity of the original solution. Thus if insulin is added to a solution of edestin and the hydrogen ion concentration is adjusted to 6·89, the iso-electric point of edestin, the precipitate carries down all the potent material originally added. Addition of copper sulphate to an acid solution of insulin caused a precipitate which contained much of the potent material. The iso-electric precipitate method is very widely used for the commercial purification of insulin.

2. *Adsorption Methods.*—The method of Moloney and

Findlay (23) has already been referred to: its success depends on the readiness with which insulin is adsorbed by benzoic acid. Insulin is adsorbed from its solutions by a variety of substances. Animal charcoal is one of these, and a recent method of purification due to the same workers is based upon this fact. It will be remembered that after adsorption of the insulin on to benzoic acid, the benzoic acid was removed by dissolving it in ether. Such a method is obviously inapplicable to the removal of charcoal, and a different principle, depending on a property of colloids, is employed for the recovery of the adsorbed insulin in this later method. This consists in replacing the insulin which is adsorbed on the charcoal by another substance, and Moloney and Findlay have successfully used fatty acids, benzoic or salicylic acids, for this purpose.

The practical details of the method are as follows: ten litres of a partially purified solution of insulin are adjusted to pH 2·5 and left overnight with 400 g. of charcoal, with occasional stirring. The charcoal is then removed, washed with water, and stirred with 2 litres of 5 per cent. acetic acid in 60 per cent. alcohol. This dissolves certain impurities, but leaves the insulin. After filtration, the charcoal is digested for several hours at room temperature with 3·5 litres of a 12 per cent. solution of benzoic acid in 60 per cent. alcohol. The insulin is recovered from the solution by evaporating off the alcohol and removing the benzoic acid with ether. The ether dissolved in the aqueous residue is removed by evaporation, and the solution then contains practically all the insulin in a state of purity. This method has been successfully applied to the commercial purification of insulin, and a method of preparation from the pancreas, based on the same principle, which promises to give good results, is being worked out by Moloney and Findlay. These authors make the interesting suggestion that the poor yields obtained in the acid alcohol extraction processes are due to adsorption of the active principle by the fatty acids liberated from the fats present in the gland through the action of the acid alcohol.

3. *The Picrate Method of Purification.*—This method has been worked out by Dudley (32). In this process the crude insulin is first dissolved in a small volume of water and the insoluble matter removed by centrifugation. The clear solution is then diluted with sufficient water to form a 1·5 per cent. solution, calculated on the weight of original material. The pH of the solution is then adjusted to about 5 by adding acid or alkali until a faint turbidity, due to iso-electric precipitation, begins to appear. A volume of saturated aqueous picric acid solution equal to half the volume of the solution is then added, causing an immediate precipitate of the picrate. After a day or two this has completely settled, and the supernatant liquid is rejected.

The moist, lemon-yellow precipitate is dissolved in the minimum quantity of cooled N/10 sodium carbonate solution to give an almost clear, brown solution, which is then filtered through a folded filter-paper. The filter-paper is washed completely by addition of distilled water, and the resulting clear filtrate diluted to contain about 1·5 to 2 g. of picrate per litre. During these operations the temperature should preferably be kept below 10° C. A quantity of N/10 hydrochloric acid equivalent to the sodium carbonate used is then added cautiously, with stirring. The picrate is immediately precipitated, and to each litre of the solution 250 c.c. of saturated aqueous picric acid solution are added. The liquid may be decanted off after a day or two, and the picrate is washed on a Büchner funnel until it is free from sodium chloride with a dilute solution of picric acid, prepared by diluting 5 c.c. of the saturated solution to 100 c.c. The moist purified picrate is then transferred to a small beaker and thoroughly rubbed with a minimal quantity of a solution of hydrochloric acid in alcohol until it is all dissolved. [The acid alcohol is prepared by mixing 25 c.c. (aqueous) 3 N hydrochloric acid with 75 c.c. absolute alcohol.] Pure acetone is then added until no further precipitate appears (10-20 volumes). The white flocculent precipitate of insulin hydrochloride settles rapidly and is immediately

filtered on a Büchner funnel, washed with acetone, and then with dry ether until free from picric acid, and is dried over sulphuric acid in a vacuum desiccator.

Physical and Chemical Properties

It is doubtful if "insulin" has yet been obtained as a definite chemical individual: indeed it is possible that there may be a whole series of substances, closely related in their properties, that are known as insulin. Or insulin may be present in all the preparations that have been made as yet as an adsorbed trace of the active substance, attached to a relatively large mass of protein. If this is so, as Somogyi, Doisy and Shaffer (33) point out, insulin must be a very remarkably active substance. It must be borne in mind, however, that this is not an impossibility. For example, one one-thousandth of a milligram of ricin per kilogram constitutes a lethal dose for a small animal.

Since the degree of activity, that is the weight of solid matter containing one rabbit unit, of the insulin used by various investigators varies between 25 mg. to something rather less than 0·1 mg., it is not surprising that very different results have been arrived at by various workers as to its physical and chemical properties. In view of this, only those points upon which the majority of recent workers are agreed will be described, and even these must be accepted only provisionally until it is shown that they apply equally to the pure substance.

As we know it at present, insulin is a perfectly white, amorphous non-hygroscopic substance. It dissolves freely in water, except at about pH 5 to 6, when it is precipitated (incompletely) from its solution. In the absence of more than traces of salts, the precipitation is between pH 4·4 to 5·8, with optimum flocking out near pH 5, which is the approximate "iso-electric point" (Somogyi, Doisy and Shaffer (33)). Inorganic salts modify this precipitation. Moderate concentrations of salts increase the solubility of the "iso-electric protein" within the precipitation zone,

but higher concentrations ($\frac{1}{3}$ to $\frac{1}{2}$ saturation with sodium sulphate or sodium chloride) cause almost complete precipitation even at acid reactions above the iso-electric range. This fact explains the success of Doisy, Somogyi and Shaffer's extraction process (24) already described.

In addition to the iso-electric precipitate at pH 5, an active precipitate is formed on the addition of concentrated hydrochloric acid to the aqueous solution until it contains about 3·3 per cent. of hydrochloric acid (Dudley (32)). It is also insoluble in 5 N sulphuric acid (Somogyi, Doisy and Shaffer (33)).

According to Shonle and Waldo (34), insulin is soluble in acidified absolute alcohol, but not in neutral or alkaline (absolute) alcohol. It is insoluble or very slightly soluble in tetrachloromethane, ethyl acetate, isobutyl alcohol, amyl alcohol, chloroform, acetone, light petroleum, ethyl ether, benzene, xylene and pyridine. It is easily soluble in glacial acetic acid, phenol, formamide and paraldehyde. Insulin dissolves in aqueous alcohol containing up to about 90 per cent. of alcohol.

Insulin is remarkably stable in acid solution. Heating to 100° C. for thirty minutes in N/10 sulphuric acid did not destroy its activity, although after one hour some destruction takes place. In alkaline solution it is much less stable, and is destroyed by N/10 sodium hydroxide in one and a half hours at ordinary temperature (Dudley (32)). Aqueous solutions of insulin are lævo-rotatory (Doisy, Somogyi and Shaffer (24)). Shonle and Waldo suggest that racemisation may take place on treatment with alkalis, but they do not adduce experimental evidence to support this. They were unable to detect any increase in the α-amino groups by means of the ninhydrin test, when insulin was inactivated by heating with alkali.

Insulin in the purest form yet obtained appears to resemble a colloid in its physical behaviour. It is readily and completely adsorbed from its solution by a variety of substances, such as benzoic acid, animal charcoal or kieselguhr.

The analyses of specimens of carefully purified insulin

by various observers show that it contains no phosphorus, but contains sulphur and about 14 per cent. of nitrogen, in addition to carbon, hydrogen and oxygen.

The Quantitative Chemical Analysis of Insulin Preparations. Shonle and Waldo (34) have made quantitative analyses of a number of insulin preparations of varying degrees of purity, as estimated by the physiological activity. They have also investigated the colour reactions on a quantitative basis, but there appears to be no correlation between the intensity of the chemical reaction and the physiological activity in the highly purified extracts.

The table shows a series of analyses by Shonle and Waldo (*loc. cit.*).

Description.	C.*	H.*	N.*	Ash.	Organic Solids.	N.
	per cent.	per cent.	per cent.	mg. per unit.†	mg. per unit.†	mg. per unit.†
"Crude insulin" precipitated from 93 per cent. alcohol.	42·60	7·47	17·44	16·50	2·2200	0·3870
	40·19	8·70	18·39	17·90	0·5460	0·1000
	39·25	6·45	13·52	19·80	1·7700	0·2390
"Crude insulin" reprecipitated at the iso-electric point until the N per unit was constant.	51·48	12·24	15·85	1·90	0·0491	0·0078
	51·15	7·77	21·85	2·45	0·0585	0·0127
As above, but also purified twice by iso-electric precipitation with trichloracetic acid.	41·12	8·68	17·95	3·66	0·0613	0·0109
An ash-free sample.	38·83	6·41	18·43	0·00	0·0337	0·0061

* Calculated on organic solids.
† These results are based on the modified Toronto unit, and therefore require to be multiplied by three to bring them into line with the units used in this book.

From this table it is pointed out by Shonle and Waldo that precipitation methods fail to give a substance of constant composition. "It is probable that we are dealing with a complex mixture of closely related substances, whose composition, during the process of re-precipitation, tends to become constant."

Attention has been drawn by Somogyi, Doisy and Shaffer (*loc. cit.* Addendum) to the high ash content of these preparations, and they also question their freedom from inactive iso-electric protein. Quite recently Cruto (37) has analysed a purified preparation of "insulin sulphate" which was free from phosphorus and gave the following results: C = 47·73; H = 7·27; N = 14·53; S = 1·73; O = 22·84; H_2SO_4 = 5·90 per cent.

Reactions of Insulin.—The purest preparations of insulin up to the present have almost all given the *biuret reaction*. Piper, Allen and Murlin (28-29), and Macleod, however, claim to have prepared extracts which did not give this reaction. Macleod obtained his material from the pancreas of the skate. Somogyi, Doisy and Shaffer (33) express the opinion that the absence of this test in these cases was due to the use of too dilute solutions of the extract.

The *Pauly reaction* is given strongly, indicating the presence of the iminazole ring (see p. 94).

The *Millon reaction* for tyrosine is faint and doubtful. *Seliwanoff's reaction* for fructose is not given, and Shonle and Waldo (*loc. cit.*) state that the *Mollisch test* for carbohydrate is negative, as are also the *Hopkins-Cole* reaction for tryptophane and *Ehrlich's p-dimethylamino benzaldehyde test* for the indole ring. Dudley (32) says that the glyoxylic test is negative with his preparations.

As already mentioned, insulin contains sulphur, but the mode of linking is uncertain. Insulin gives a positive result with *Folin and Looney's* test for cystine and tyrosine, and for reduced sulphur after boiling with sodium hydroxide. It gives no colour with sodium nitroprusside (no SH groups). Ammoniacal silver nitrate gives no precipitate (absence of purines). Ehrlich's diazo-reaction for tyrosine and histidine is given after destruction of the tyrosine by nitration, indicating the presence of histidine (Shonle and Waldo (34)). Most preparations of insulin have given a positive xanthoproteic test indicating the presence of the benzene nucleus.

Shonle and Waldo point out the necessity for using a

concentrated solution for the majority of these tests. They used a solution containing 100 units in 1 c.c.

Insulin is precipitated from its solution completely by protein precipitants, such as tungstic, phosphoric, nitric, trichloracetic, tannic, picric and metaphosphoric acids: absolute alcohol, sulphates of sodium, zinc and ammonia. The whole of the activity seems to be carried down with the precipitate, but repeated reprecipitation causes loss of activity, for the yield diminishes although the solution after precipitation does not appear to contain active material. Oxidation with very dilute hydrogen peroxide or potassium permanganate rapidly destroys the activity.

Reduction with sodium bisulphite, hydrogen or stannous chloride also destroys the activity (36). All attempts to reactivate insulin thus inactivated have failed.

The only active derivatives that have been examined are the so-called "hydrochloride", the "picrate" (Dudley (32)) and the sulphate (Cruto (35)). The properties of these substances, their interconversion and formation from insulin have already been described. Insulin does not appear to lose its activity in the presence of moderate concentrations of formaldehyde (31) or of tricresol. The latter substance is used as an antiseptic in the preparations of solutions for clinical use.

Incubation with trypsin or pepsin rapidly destroys the activity. The following experiments illustrate this (Dudley (32)): 0·5 g. of commercial trypsin was dissolved in 10 c.c. of N 10 sodium carbonate solution, and 20 mg. of insulin (rabbit unit 10 mg.) was dissolved in 2 c.c. of water. This was divided into two portions of 1 c.c.: one was incubated for two hours with 1 c.c. of the trypsin solution, and the other for two hours with 1 c.c. of the same solution after its tryptic activity had been destroyed by heating at 100° C. for thirty minutes. The following table shows the effect of these solutions on the blood-sugar of two rabbits (Dudley, *loc. cit.*):

Blood-sugar.	Trypsin Experiment.	Heated Trypsin Control.
Normal	·092 per cent.	·101 per cent.
1 hour after injection . .	·104 ,,	·059 ,,
2 hours ,, ,, . .	·101 ,,	·066 ,,
3 hours ,, ,, . .	·104 ,,	·074 ,,
Rabbit weight	3·5 kg.	3·25 kg.

The activity has been completely destroyed by incubation with trypsin, and the same applies to the action of pepsin.

These experiments, taken in conjunction with the physical properties and the complete precipitation from aqueous solution by picric acid, have caused Dudley to express the belief that insulin is a very complex substance, protein-like in its nature. "The hope of its isolation as a chemically pure substance becomes slender, and of its synthesis very remote, by methods at present at our disposal" (*loc. cit.*).

Doisy, Somogyi and Shaffer (24) state that insulin appears to be either an albumose or a globulin. In their later paper (33) they confirm the view that insulin seems to be a protein. "Although we feel that our data alone are insufficient to establish the identity of insulin, we have been strongly impressed by the quantitatively very similar, if not identical, activity of our different 'pure' preparations of 'insulin protein', purified by different methods, and with the fact that these preparations appear to have otherwise the same properties" (Somogyi, Doisy and Shaffer, *loc. cit.*). The indifference of insulin to treatment by formalin (Dodds and Dickens (31)) in moderate concentration appears to be contrary to these views.

Shonle and Waldo (34) conclude that the pancreatic substance containing insulin appears to be a complex mixture of proteoses which give typical protein reactions. "Further research must determine whether the active principle is a proteose or merely intimately associated with a proteose fraction."

Witzemann and Levshis (36) postulate a peptone-polypeptide type of insulin and a protein type. Their

arguments are based on the differences in solubility of crude and purified insulin.

It will be evident that there is no settled view upon the chemical nature of insulin at the present time, and it must be left to the results of further research before any opinion can be put forward on the subject.

Physiological Action

The first action of insulin to be observed was its *blood-sugar lowering effect*. There are many substances possessing this property, but the hypoglycæmia due to the injection of insulin is quite characteristic. Whereas other substances require a considerable time to produce their effect, insulin commences to act almost at once. The blood-sugar content falls steadily, the amount and duration of the depression being proportional to the dose.

Allan (37) has shown that the relation between the dose and the extent of the fall is a logarithmic one. This is also true for the diabetic dog. One of the reasons for this lies in the fact that large doses of insulin are attended by an excretion of a large part of it into the urine.

The blood-sugar content starts to decrease within a few minutes of the injection, and the lowest point may be reached after one to two hours, or even later, according to the dosage. One of the most interesting features is that when the blood-sugar content is very low the animal frequently develops a peculiar condition, associated with a series of signs differing according to the species. Thus, in the rabbit, convulsions usually appear when the sugar content of the blood has fallen to 45 mg. per 100 c.c. or thereabouts. The first symptom to appear is usually the loss of muscular tone, with the result that the rabbit lolls over on its side, or it lies stretched full length on the floor. An attempt to right itself is followed by immediate clonic convulsions, with opisthotonus when at the height of the convulsion. The spasms occur fairly rapidly, with the result that the rabbit seems to turn round and round on the floor. After a few seconds the

spasm passes, and the animal may sit up in an apparently normal condition, when, after a short while, another attack supervenes. Attempts at movement or touching the rabbit may start the seizure. The convulsive attacks gradually grow weaker and weaker, the temperature falls, and if a large dose has been given the animal finally dies from respiratory failure. Rigor mortis appears within a few minutes, and the animal becomes as stiff as a board long before it is cold. Towards the end, a condition of coma very frequently exists. The hypoglycæmic reaction differs in different animals. Thus in the cat great excitability, salivation, mewing and relaxation of the sphincters are the most important symptoms. In dogs Macleod (38) says that excitability, barking and frothing at the mouth are most prominent. Muscular twitchings and relaxation of sphincters occur early, whilst convulsions similar to those in rabbits also occur. Mice show little response to insulin at room temperature, but August Krogh (39) has shown that convulsions, paralysis of the hind legs and coma appear if the animals are kept in an incubator at 28° C. At room temperature frogs seem to be immune to the effects of insulin. In man excitability, sweating and salivation, together with various vasomotor disturbances such as flushing and pallor, are usually evident. Loss of consciousness may result if the symptoms are not relieved, but definite convulsions have never been noted by any observers up to the present.

In rabbits these symptoms of hypoglycæmia usually appear when the concentration of blood-sugar is about 45 mg. per 100 c.c. They may occur at higher or lower figures, however.

With regard to man, the onset of symptoms may take place when the blood-sugar content is below 75 mg. per 100 c.c., whilst cases have been described in which the blood-sugar fell to 32 mg. per 100 c.c. without the onset of symptoms (Banting (40)). Leyton (41) has stated that symptoms may occur at a much higher figure if the hyperglycæmia of a chronic diabetic is suddenly reduced. Thus in one of his cases symptoms appeared when the blood-

sugar fell to 250 mg. per 100 c.c. Langdon Brown (42) has also called attention to this point, and described the onset of symptoms in a similar case when the blood sugar was 150 mg. per 100 c.c. A full consideration of the diagnosis and treatment of this condition is outside the scope of the present volume.

The hypoglycæmic state can be controlled by various methods, and since each of these bears upon the theoretical considerations, it will perhaps be advisable to deal with them separately.

They may be tabulated as follows :

I. By the administration of glucose and certain carbohydrate derivatives.
II. By the administration of adrenaline.
III. By the administration of pituitrin.

I. *Reduction of Hypoglycæmia by the Administration of Glucose and certain Carbohydrate Derivatives.* That convulsions were associated in some way with a reduced blood-sugar content was shown in 1921 by Mann and Magath (43), working upon the effects of excision of the liver. Administration of glucose intravenously relieved the symptoms in these animals almost immediately. The rapidity and permanence of the cure naturally depend upon the severity of the symptoms, and upon the quantity of glucose injected. Macleod uses 2 g. of glucose per kg. of body weight, injected either subcutaneously or intravenously in normal saline. In most cases the recovery is dramatic, taking place in a few minutes. The animal is usually very hungry on its return to consciousness, and, if allowed to eat, goes to sleep and wakes up none the worse. If too small an injection is given, the animal's condition relapses. Even when recovery is taking place, one of the signs may be an increase in the strength of the convulsions.

Since glucose is only one of a series of bodies with very similar constitutions, the effects of various other sugars have been studied. Noble and Macleod (44) published an account of such an investigation, but a much fuller

series of compounds was tested later by Herring, Irvine and Macleod (45). The results of this extremely important paper are summarised below : glucose is far more effective than practically all other carbohydrates, whilst it may be formulated as a general rule that those monosaccharides which are most easily fermented by yeast are the most potent. This, therefore, would include glucose, fructose and mannose. The power of counteracting hypoglycæmia may be summarised in the following table. Mice were used for the standardisations.

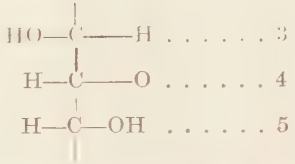
Carbohydrate Derivative.	Potency for relieving Hypoglycæmic Symptoms.
Glucose	Most potent.
Mannose	Potent.
Fructose	Potent. May relapse.
Galactose	Poor. Cannot replace glucose.
Lactose	Nil.
Sucrose	Nil.
Maltose	Potent, but slow.
2, 3, 5, 6 tetramethyl glucose	Inactive.
2, 3, 5 trimethyl glucose	
Tetra-acetyl fructose and β-methyl glucoside	
Tetramethyl β-methyl glucoside	
Tetramethyl γ-methyl glucoside	
Glucose monoacetone	
Mannitol	
Dulcitol	
β-glucosan	
Salicin	? Very slight potency.

Irvine, in the paper under consideration, discusses the results from the point of view of structure. For full details the original paper should be referred to. He draws attention to the following points :

1. All compounds without a reducing group are inactive, consequently this group is regarded as essential. Even the α- and β-methyl glucosides, where the reducing group is modified to the minimum, are inactive.

2. Certain asymmetric systems are essential, while others are not. Thus in glucose, the grouping round the second carbon atoms is H—C—OH, and in mannose is OH—C—H, *i.e.* the reverse. It would appear, therefore,

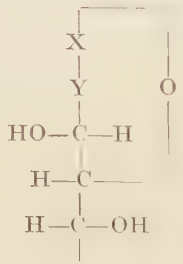
that the configuration of these groups is immaterial. Since in most of the substances tested the terminal primary alcohol group (No. 6) is unsubstituted, whereas in maltose it definitely is, it would appear that this grouping is of no importance. This narrows the discussion down to Nos. 3, 4 and 5. The state of affairs in glucose is as follows:



This grouping exists in mannose and maltose. There is no evidence of the importance of 3, but, from the results already quoted, it would appear that 5 is of importance, since in lactose, which is inactive, the ring formation is through this particular atom. Galactose, in which the ring starting from 4 is on the opposite side to glucose, is much less active than glucose, consequently it can be agreed that the grouping around 4 is of importance.

3. It would appear that both a reducing group and the grouping of 3, 4 and 5 outlined above are essential, since either alone is inactive.

4. No exception can be found " to the generalisation expressed in the statement that the type of carbohydrate molecule functional in eliminating the convulsion symptoms, occasioned by the administration of insulin, is:



5. The chemical changes taking place on injecting glucose are probably twofold. The first may consist in

an attack upon the reducing group, which would make further rearrangement of CHOH groups possible. The remaining OH groups may then react according to their position and arrangement.

6. The possibility of glycogen possessing the property of relieving the symptoms before hydrolysis to glucose is unlikely, since β-glucosan ($C_6H_{10}O_5$) is inactive. Glycogen itself could not be used, owing to its difficult solubility.

It would appear, then, that a carbohydrate must have a certain structure before it is capable of relieving convulsions.

II. *Reduction of Hypoglycæmia by the Administration of Adrenaline.*—Banting, Best, Collip, Macleod and Noble (46) showed that insulin counteracts the hyperglycæmia due to injections of adrenaline, and consequently it would appear that the two hormones are antagonistic. This fact was proved by the above investigators, working with Eadie, and was made the basis of a method of standardisation. By varying the simultaneous doses of insulin and adrenaline, a state of affairs could be reached in which the blood-sugar remained more or less constant. Since it is generally accepted that adrenaline hyperglycæmia is due to increased glycogenolysis, the action of insulin in this respect might be twofold :

(*a*) By preventing this glycogenolysis ; or
(*b*) By removing the sugar from the blood as fast as it is formed.

That the second of these possibilities is bound to play a part cannot be denied, since insulin will relieve hyperglycæmia due to any cause. It would appear from certain evidence that the first possibility also operates, since Macleod and Noble noticed that the hepatic glycogen in a series of animals injected with insulin plus adrenaline was much higher than in a series receiving adrenaline alone. The mechanism is therefore twofold.

McCann, Hannon and Dodd (47), and Lyman, Nichols and McCann (48) have stated that adrenaline will cause a

temporary inhibition of the convulsions, but do not recommend this as a line of treatment.

III. *Reduction of Hypoglycæmia by the Administration of Pituitrin.*—Burn (49), early in 1923, reported a series of investigations upon the interaction between pituitrin and insulin. Knowing that pituitrin possessed the property of reducing the hyperglycæmia due to anæsthesia and adrenaline, he expected to find that this substance would intensify the action of insulin. "So far from intensifying the fall produced by insulin, a simultaneous hypodermic injection of pituitary extract either reduces, or abolishes, or replaces by a rise of blood-sugar, the fall due to insulin." This action is not given by extracts of any other tissue prepared in the same way, and is not given by histamine, and, moreover, the quantities of pituitrin employed were such as to give only a very slight rise in blood-sugar. Extracts of the anterior lobe are entirely without action, whilst the activity of the posterior lobe in this respect can be destroyed by treatment with N alkali. Convulsions rapidly disappear after an injection of pituitrin, and the blood-sugar content rises.

Burn also noted that if ergotoxin be injected intravenously, the effect of a subsequent injection of insulin is intensified. Although this worker points out that no definite explanation of these results can be given, he feels that "the antagonism of pituitary extract for insulin is of a more direct and specific nature (than that of adrenaline), and is not shown by extracts of the other tissues which have been examined".

Winter and Smith state that parathyroid (Parke Davis), when administered prior to the injection of insulin, greatly increases the effect, and that a third to a quarter of the ordinary dose is sufficient to produce convulsions.

A very important investigation has been reported by Burn and Marks (50). These authors state that removal of the thyroid greatly increases the sensitivity of an animal towards insulin. Prior to their investigations similar results had been reported by Bodanski and by Duchenau, but their observations had not been fully

controlled. Burn and Marks performed very careful controls, observing the response to insulin before and after thyroidectomy on the same animals. They hold that thyroidectomised rabbits behave much more uniformly, and that it is quite possible that the thyroid is very largely responsible for variations in the reaction of rabbits.

Before proceeding to a discussion of the various theories as to the action of insulin, it will be as well to describe some of the other effects.

As already stated, insulin will reduce hyperglycæmia due to any cause.

Asphyxial forms are prevented or reduced, and the same is true of the toxic varieties, *e.g.* due to ether (Macleod (51)). Stewart and Rogoff (52) have also demonstrated that the hyperglycæmia due to the injection of morphine yields to insulin, which does not alter the other symptoms. The explanation seems to lie in the fact that the increased blood-sugar is due to the liberation of adrenaline.

The injection of insulin is followed by certain changes in the phosphate balance in the body. Thus Wigglesworth, Woodrow, Winter and Smith (53), 1923, and Winter and Smith (54), 1924, state that the injection of insulin is followed by a rapid fall in the inorganic phosphorus in the blood, and that a low level may be maintained long after recovery by glucose. The total acid soluble phosphorus is little affected. The explanation may lie either in an increased phosphorus excretion, a theory which these workers do not favour, or in a possible combination with the glucose, or the conversion into a complex organic phosphorus compound precipitated with the proteins. Sokhey and Allan (55) noted that the injection of insulin was followed by a diminution in the excretion of inorganic phosphorus in the urine. Similar results were obtained by the administration of sugar alone, and of sugar and insulin together. These authors suggest that their result could be explained in various ways, but postpone any definite statement.

Kasuhara and Uetani (56) showed that the reducing power of the cerebro spinal fluid was also reduced under insulin administration. Winter and Smith (*loc. cit.*) state that they obtained inhibition of an isolated strip of intestine, and that this action took place through a depressor action on the vagal nerve endings. It has been tentatively suggested by Needham, Smith and Winter (57) that insulin might cause glucose to be converted into inositol. They state, however, that their evidence is very inconclusive.

Haldane, Kay and Smith (58) state that insulin causes an increase in the blood volume, the osmotic pressure remaining constant. This observation is of importance in the calculation of the distribution of substances between corpuscles and plasma, for if the increase in volume following insulin injection is not allowed for, error will result.

Injections of insulin cause a fall in blood pressure, as do all tissue extracts. Dudley has shown that his method of purification removes this depressor substance. Collip (59) noted the presence of acetone bodies in the urine of hypoglycæmic rabbits. He regards this as due to an acidosis.

Effects upon Metabolism.—A series of isolated facts concerning the action of insulin upon the metabolism of various substances has been given, but as yet no attempt has been made to form a theory of the general action of this body. The pharmaco-dynamics may be summed up by saying that the blood-sugar is greatly reduced after injection. The problem resolves itself into what changes take place to produce this phenomenon. Sugar disappears; where does it go? The possibilities can be classified as follows.

I. Insulin might cause the sugar of the blood to disappear without the intervention of any tissue other than the blood, *i.e.* the action could take place *in vitro*.

II. Insulin might stimulate the glycogenetic powers of the liver, with the result that sugar would be removed from the blood, to be stored as glycogen in the liver.

III. The blood-sugar reducing effect might be due to an increased combustion of glucose.

IV. Sugar might be converted into some non-reducing substance, such as fat or an unknown body.

It will be as well to take each of these possibilities in turn, and to discuss the evidence for and against each.

I. With regard to the first possibility, one of the earliest experiments performed by the Toronto workers was to study the effect of insulin upon the phenomenon of glycolysis outside the body.

Eadie, Macleod and Noble (60) were unable to find any change in the rate of disappearance of sugar from blood after the addition of insulin. Glycolysis proceeded at a normal rate. The addition of extracts of other tissues, such as muscle, and defibrinated blood and leucocytes to an insulin-glucose mixture makes not the slightest difference.

It is useless, therefore, to seek an explanation for the action of insulin along this line.

II. Investigation of the second possible explanation for the disappearance of glucose is a difficult matter, since the diabetic animal will behave differently from the normal. Injection of small quantities of insulin into a depancreatised dog causes storage of glycogen in the liver, thus appearing to support the theory. On the other hand, a normal animal behaves in exactly the opposite manner. The administration of insulin to a diabetic animal merely restores its normal function.

McCormack, Macleod and O'Brien (61) performed a series of experiments to investigate the effect of insulin administered to glycogen-free normal animals. Insulin and glucose were given to the animals, which were afterwards killed, and glycogen in the liver was estimated. A series of controls was performed, and it was found that the formation of glycogen in the liver was much greater in these than in the animals receiving insulin. Dudley and Marrian (62) performed similar experiments, using animals with a good store of glycogen, ensured by feeding with carrots for two days previous to the experiments.

Exactly similar results to the above were found, namely, that the glycogen content is decreased in those animals receiving insulin. Dudley and Marrian (62) also pointed out that no glycogen may be present in the muscles, but a considerable amount may be present in the liver. Macleod also advances a further series of arguments against this glycogenetic hypothesis.

1. Sugar disappears much more rapidly after insulin than after removal of the liver.

2. Removal of the liver makes no difference to the rate of action of insulin.

3. Post-mortem glycolysis is unaltered by insulin.

It would appear, therefore, that insulin accelerates the breaking down of glycogen.

The exact mechanism of this action is difficult to explain. It might lie in a direct stimulation of glycogenolysis, or act indirectly by lowering the blood-sugar content. Noble and Macleod (63) attempted to elucidate this point by a series of experiments with a surviving turtle's liver, which, by reason of its anatomy, is especially suited to such experiments.

They found that perfusion with a sugar solution increased the hepatic glycogen, and that the addition of insulin made little or no difference, either to the percentage of glycogen in the liver or to the output of glucose.

These observations would tend to show, therefore, that insulin acts by causing a reduction in the blood-sugar, which is followed by a compensatory glycogenolysis.

No explanation for the blood-sugar reducing action of insulin can be found under this heading.

III. The glucose might be removed by increased oxidation. Some of the first observations performed on the action of insulin were to investigate this point. The Toronto workers, with Pember and Dixon, showed that the administration of insulin was followed by a rise in the respiratory quotient, in both normal and diabetic animals. In some cases the figure was above unity. Macleod explained these facts by stating that an increased combustion of carbohydrate occurred, together, possibly, with

a conversion into fat, similar to the conditions in hibernating animals prior to the winter.

Dudley, Laidlaw, Trevan and Boock (64), working with mice, observed the opposite effect, and noticed a depression of metabolism following insulin administration. Oxygen consumption and carbon dioxide excretion were slowed down, but occasionally the respiratory quotient rose, probably due to a more rapid decrease in the oxygen intake. Kellaway and Hughes (65) found a rise in the respiratory quotient of normal starving individuals after the injection of insulin, but the total exchange was not sufficient to account for any marked increase in carbohydrate metabolism. Macleod and his co-workers reinvestigated these points, taking great care to avoid the influence of any factors other than those under inquiry. They found that the respiratory quotient rose during hypoglycæmia, but that the interpretation of their results was rendered difficult owing to the added effect of the muscular contractions during convulsions. The majority of workers hold the view that there is an increase in the respiratory quotient, but that the increased respiratory metabolism is not sufficient to account for the reduction in blood-sugar.

The work of Winter and Smith can best be discussed under the present heading. In 1920 Hewitt and Pryde (66) published an account of some polarimetric methods of investigating the effect of intestinal mucosa on glucose solutions. They stated that after contact with the mucosa a glucose solution showed an upward mutarotation, and concluded that whilst the contact was being made there must have been a downward mutarotation. They explained this as being due to the formation of an unstable, highly reactive isomeride, γ-glucose. Stivens and Reid (67), repeating these observations in 1923, were unable to detect any such changes as described by Hewitt and Pryde. In the same year Winter and Smith (68) advanced a similar hypothesis in connection with the blood-sugar. They stated that the blood-sugar of normal individuals could not be the ordinary α-β-variety, since the initial

rotatory power was low as compared with the copper reducing value. In diabetic blood they found that the rotatory and reducing powers corresponded. They therefore concluded that the sugar of normal blood differed from that of diabetic blood, and were led to suggest that the substance of low rotatory power was γ-glucose, following the views of Hewitt and Pryde. Their theory as to the causation of diabetes was that normally glycogen is broken down to α-β-glucose, and, before metabolism, this mixture must be changed to γ-glucose. Diabetes would be due to the absence of some specific substance capable of bringing about this change, since only the γ-variety can be oxidised in the body. Later Forrest, Smith and Winter (69) suggested that insulin was responsible for this change. It is also stated that incubation of glucose solutions with insulin and liver extracts is accompanied by an alteration in the rotatory power, the reducing power remaining constant. This work has been repeated by a number of investigators, who fail to confirm it. Thus Eadie (70) was unable to demonstrate such changes. Van Creveld (71) also failed to confirm this work, and agreed with Stivens and Weymouth Reid (72) with regard to the investigations of Hewitt and Pryde in not finding the changes described by them.

A very neat experiment was performed by Tallerman (73), who pointed out that the difficulties in dealing with blood could be overcome by employing the urine of normal men after an injection of phloridzin. It is reasonable to suppose that the resulting sugar in the urine would of necessity be of the same type as in the blood. He proved this to be the ordinary α-β-variety. All observers have called attention to the severe treatment of the blood prior to the polarimetric observations, and the explanation of Winter and Smith's results might lie in this (Eadie). Moreover, as Macleod points out, the differences in the readings are so extremely small that other explanations might be advanced. Irvine (74), writing on these questions, says, "There is little prospect of detecting these fugitive isomerides by ordinary processes." It

would also appear, even assuming that the theories advanced by Winter and Smith are correct, that little advance is made in the broad issue, since it has been shown that the action of insulin cannot be explained by oxidation alone.

IV. By a process of exclusion, we are driven to adopt the somewhat indefinite fourth possibility, namely, that insulin causes glucose to be converted into some non-reducing, unknown substance. As to the nature of this substance, nothing is known. Pember and Dixon (75) suggested that fat might be formed. This, however, has been negatived by the work of Dudley and Marrian (*loc. cit.*), who were unable to detect any alteration in the amount or iodine value of the fatty acids in the livers of mice following injections of insulin.

A very important question arises as to where this conversion of glucose takes place. It has been shown that neither the brain (Olmsted and Logan (76)) nor the liver (Mann and Magath (77)) is essential to the action. With regard to the rest of the tissues, two very important papers have been published concerning these points. The first is that of Hepburn and Latchford (78), who worked with isolated mammalian hearts. They showed that the addition of insulin to the perfusion fluid greatly accelerates the removal of glucose from the solution. If this is true of heart muscle, it is very probably true of other tissues. The second paper is a description of an exhaustive series of investigations performed by Burn and Dale (79). They repeated and confirmed Hepburn and Latchford's work with the surviving heart, and, in addition, measured the carbon dioxide production during the experiments. They found a slight increase in carbon dioxide production with insulin, but not anything like enough to account for the acceleration in the rate of removal of glucose. They also investigated a series of hearts obtained from depancreatised cats, and found that they behaved similarly to the normal organs. The next experiments were performed upon decapitated and eviscerated cats, with a view to determining the site of these changes. Prepara-

tions were made in which various viscera were precluded, by suitable arrangements, from participating in the effect. They came to the conclusion that only the muscles and the heart were essential. This work renders untenable the theory that glucose must first be converted into a reactive form by the liver, since injection of ordinary glucose into a preparation is followed by immediate disappearance. As they point out, "this does not exclude the possibility that other organs, such as the liver, may be equally concerned in this process, as seen in the whole animal".

Immediate conversion into fat or lactic acid seems improbable. Campbell and Dudley (80) described some experiments in which the oxygen tension in various tissues was studied by means of introducing air in their vicinity, it having been shown previously that the oxygen tension in the air varies with that in the tissues. They showed that the oxygen tension falls as the blood-sugar decreases. This effect was most marked under the skin and in the region of muscles. There was no fall in the oxygen tension of air similarly introduced into the abdominal cavity. This fall in oxygen tension is explained as being due to the utilisation of oxygen. Again it is far too small to account for the disappearance of anything but a very small quantity of glucose. There is usually an increase of carbon dioxide tension in these regions.

In spite of the vast amount of work performed upon the physiological action of insulin, the position is still very uncertain. The present view may be summed up by stating that the administration of excess of insulin concentrates metabolism on the carbohydrates, thus sparing an equivalent of protein and fat (Dale). In addition, there is the conversion of glucose into an unknown substance. All tissues take part in these processes, but it seems probable that the heart and muscles are the essentials to the phenomenon.

The Standardisation of Insulin

The only methods of standardisation of insulin which are available are based upon its action on the blood-sugar. Since the nature of insulin is as yet undetermined, chemical methods cannot be used for the standardisation. Moreover, if the views of Dudley and of Somogyi, Doisy and Shaffer as to the protein-like nature of insulin are correct, it is doubtful if chemical methods will be applicable to the problem until our knowledge of proteins is much more profound than it is to-day.

The information upon which the physiological methods of standardisation are based is largely due to the work of Banting, Best, Collip, Macleod and Noble (81), who studied the effect of injections of insulin on the blood-sugar of normal rabbits. These investigators found that subcutaneous injection of insulin caused a fall in the percentage of blood-sugar within a few hours, accompanied by signs of hunger and thirst, hyperexcitability and apparent fear. The rabbit may recover from these earlier symptoms, but frequently the hyperexcitability becomes extreme, and convulsions involving the whole body and lasting several minutes supervene. In the majority of cases of rabbits exhibiting convulsions, the blood-sugar was found to be in the region of 0·045 per cent. Subcutaneous (or intravenous) injection of glucose relieved the symptoms. In this paper the authors suggested the use of a unit which is known as the " Original Toronto Unit ", which is defined as the quantity of insulin which causes the blood-sugar of normal rabbits to fall to 0·045 per cent. within four hours.

Later, McCormick, Macleod, O'Brien and Noble (82) described certain precautions which must be applied in making the assay by this method. These points are as follows :

1. The animals should be kept without food for twenty-four hours preceding the test.
2. They should be all of nearly the same weight.

3. Three animals should be used for each test.
4. The blood sugar should be examined one and a half and again three hours after the injection.

If one unit is considered as the amount of insulin capable of reducing the blood sugar to the convulsive level (0·045 per cent.) within three hours, and this is not reached by any one of the animals, a second assay on another group of animals should be made with double the previous dose. If too much insulin is given in the first trial the assay should be repeated, half the previous dose being used.

Unfortunately, much confusion has been caused by alterations in this original unit. It was thought that this unit was too large and that very mild cases of diabetes might require fractional doses. For this reason a new unit was described which is equal to one-third of the original unit, and, more recently, a 40 per cent. increase upon this value has been announced. This unit is in common use in America to-day. Other workers have used rabbits of 1 kilogram body weight for the standardisation. In order to avoid confusion we have decided to use throughout this book the "Original Toronto Unit", unless otherwise stated. This unit may be defined as the quantity of insulin which is required to lower the blood-sugar of a 2 kg. rabbit which has previously been starved for sixteen to twenty-four hours, from a normal value of about 0·11 per cent. to 0·045 per cent., in a period of from two to four hours.

The assay is performed in the following manner. The insulin to be standardised is weighed and dissolved in an appropriate volume of water. The insulin obtained by the majority of the processes which have been described varies in activity between narrow limits, and a rough estimate of the activity can usually be formed before standardisation. In this case it should be dissolved so that 1 c.c. of solution has about one rabbit unit. Clinical solutions of insulin require to be diluted to about this concentration before standardisation.

If one has no idea of the activity of the preparation to be standardised, it is necessary to prepare solutions of various dilutions, and to perform preliminary standardisations on these before proceeding to the more accurate assay.

The rabbits are starved from mid-day on the day before standardisation, and samples of blood are taken for analysis the following morning from an ear-vein. Three rabbits at least should be used. Their weights should not differ much from 2 kg. The dose is approximately proportional to the body weight if the latter does not differ much from 2 kg. (Fenger and Wilson (25)) (Sansum (83)). It is preferable to use rabbits the response of which to insulin is already known from previous tests. The use of excitable rabbits should be avoided. Immediately after taking the samples of blood, the insulin solution is injected in doses, some of which should be slightly above and below the estimated dose. Subcutaneous injection is usually employed for this purpose.

Samples of blood are taken after each hour up to the third after injection, and blood-sugar estimations are performed on all these samples of blood. It is then a simple matter to find which is the smallest dose to produce a lowering of the blood-sugar to about 0·045 per cent. The lowerings produced by the other doses should be roughly proportional to the size of the dose, but it is necessary to emphasise the fact that the method is by no means an accurately quantitative one, owing to the variations in the responses of different rabbits to the same dose of insulin.

The following example will serve to illustrate the method, although no exact quantitative value must be attached to the figures quoted. One gram of a preparation, the rabbit unit of which was known to be in the region of 1 milligram, was dissolved in 20 c.c. of water. This solution therefore contains 50 mg. per c.c. After thorough mixing, 1 c.c. of the solution was withdrawn with a pipette and diluted to 50 c.c. This diluted solution now contains 1 mg. per c.c. Three rabbits were used for

the test (rabbits A, B and C), and doses of 0·8, 1·0 and 1·2 c.c. were given. The blood-sugar estimations are given in the table :

Rabbit.	Dose.	Normal.	Blood-sugars.		
			After 1 Hour.	2 Hours.	3 Hours.
A	0·8 c.c.	0·113	0·083	0·059	0·060
B	1·0 c.c.	0·125	0·065	0·044	0·049
C	1·2 c.c.	0·107	0·057	0·040	Convulsed violently. Glucose given.

The rabbit-dose of this substance would be taken as 1·0 c.c. This solution contains 1 mg. per c.c. The rabbit unit is therefore 1 mg. Animals other than rabbits have been used for the testing of insulin. The principal alternatives are the mouse and rat.

The Mouse Method.—Starved white mice or rats are very susceptible to the action of insulin injected intraperitoneally (Fraser (84)). The effect of the injection is greatly influenced by the temperature (Voegthin and Dunn (85)), and for accurate results it is necessary to keep the animals in chambers, the temperature of which is controlled by a thermostat at about 28° C. The method is similar to that used for rabbits, but blood-sugar estimations are not performed, the standardisation being based upon the minimal convulsive dose, or else upon the maximum dose upon which no mouse convulses. The latter is the more accurate criterion, but in any case it is necessary to repeat the standardisation upon another series of mice to confirm the result. The relation between the sizes of the mouse and the rabbit units is not accurately known at present.

In order to make the section on the standardisation sufficiently complete, the following method of blood-sugar estimation has been included.

The Folin and Wu Blood-sugar Method (86).—In preparing the filtrate for this method two solutions are necessary.

(*a*) 10 per cent. sodium tungstate. Great care should be taken to ensure that the tungstate is pure.
(*b*) $\frac{2}{3}$ N sulphuric acid.

The filtrate is prepared as follows :

One volume of blood.
Seven volumes of water.
One volume of solution (*a*).
One volume of solution (*b*).

The flask is shaken vigorously ; complete precipitation of the blood proteins is indicated by a change of colour from bright red to a chocolate brown, and there should be no bubbles. The resulting solution is filtered, using a minimum quantity of filter-paper, and pouring the whole of the fluid on to the funnel at once.

Incomplete precipitation of the blood proteins will give a cloudy filtrate. By adding a drop of 10 per cent. sulphuric acid, and at the same time shaking vigorously, it may be possible to save the specimen, but this is not recommended. To estimate the blood-sugar by this method the following three solutions are required :

1. Standard sugar solutions.
2. Alkaline copper solution.
3. Molybdic acid solution.

These solutions are prepared as follows :

1. One gram of pure glucose is dissolved in 50 c.c. of 0·25 per cent. benzoic acid solution in water. This solution is then transferred to a 100 c.c. volumetric flask, and is made up to the mark with benzoic acid solution. One c.c. of this solution made up to 100 c.c. will serve as a standard for most specimens of blood, but it is well to have a double standard ready in case a very high blood-sugar is encountered. This is made by diluting 2 c.c. of the stock solution to 100 c.c. (all dilutions are made with the saturated benzoic acid solution). These solutions contain 0·1 and 0·2 mg. of glucose per c.c.

2. Forty grams of anhydrous sodium carbonate are dissolved in 400 c.c. of water, and the resulting solution

is transferred to a litre flask, 7·5 g. of tartaric acid are added, and when this has dissolved, 4·5 g. of crystalline copper sulphate are added. After thorough shaking the substances dissolve, and the volume is made up to a litre.

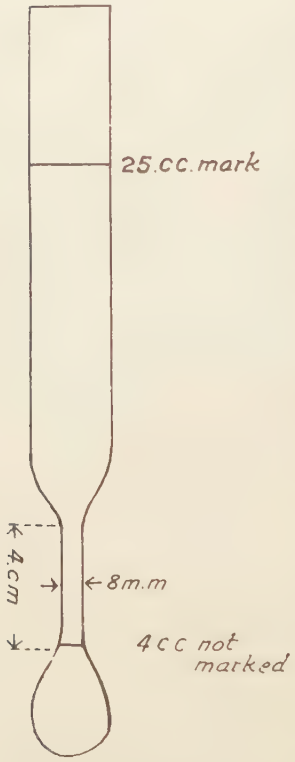


Fig. 1.—Blood-sugar tube for the Folin - Wu method. (Copied from *A Laboratory Manual of Biological Chemistry*, O. Folin. Appleton, 1923.)

3. Thirty-five grams of molybdic acid and 5 g. of sodium tungstate are placed in a litre beaker, together with 200 c.c. of 10 per cent. sodium hydroxide and 200 c.c. of water. The contents of the beaker are then boiled for half an hour or more to remove the ammonia from the molybdic acid. After cooling, the solution is transferred to a 500 c.c. measuring flask and diluted up to 350 c.c.; 125 c.c. of 85 per cent. phosphoric acid are then added, and the volume is made up to 500 c.c.

Method.—Two c.c. of the blood-filtrate and 2 c.c. of the standard glucose solution are placed in separate special sugar tubes (see Fig. 1). Two c.c. of the alkaline copper solution are added to each, and the tubes are immersed in a boiling water bath for six minutes. They are then removed and cooled in water. Two c.c. of the molybdic acid solution are added to each and, after shaking, the volume is made up to the 25 c.c. mark with water. After mixing, the solutions are compared in the colorimeter, the standard being set at a depth of 20 mm. For the low standard solution 2000 divided by the colorimetric reading (for the high

standard 4000) gives the sugar content in mg. per 100 c.c. of the original specimen.

The Yield of Insulin from Various Sources

As has already been stated, the rabbit units referred to are the original Toronto units in every case.

(*a*) *Mammalian Pancreas.*—In the earlier experiments the activities of the extracts were estimated by the extent to which they relieved the glycosuria of diabetic dogs, and quantitative estimates of the yields are difficult to make in terms of the rabbit unit.

In the earlier Toronto experiments (1922) yields of from 5 to 13 rabbit units per kg. of pancreas were obtained (*vide* Best and Scott (27)). The method of Collip involving extraction by plain alcohol yielded on the average approximately 88 rabbit units from a kg. of pancreas. Extraction with acid alcohol, by the method of Doisy, Somogyi and Shaffer (24), increased this yield to 257 units, according to the observations of Dudley (32). Fenger and Wilson (25) have used Doisy, Somogyi and Shaffer's method to estimate the insulin content of the pancreas of domestic animals. The table reproduces their results :

Animal.	Average Weight of Pancreas.	Average Rabbit Units per kg.
Cattle . . .	308·0 g.	595
Hog . . .	60·0 g.	574
Sheep . . .	18·8 g.	613

Dudley's bicarbonate process of extraction (32) gave an average yield of 412 rabbit units per kg. of pancreas. The process of Best and Scott (27) resulted in yields of about 133 units per kg., although in several experiments as much as 300 units were obtained. The recent experiments of Somogyi, Doisy and Shaffer (33) have given yields of from 500 to 830 rabbit units per kg. This is the best yield hitherto obtained by alcoholic extraction.

Moloney and Findlay (87), experimenting on the adsorption of insulin from pancreas by alcoholic benzoic acid solutions, have obtained yields of 1600 units per kg.; but the crude product obtained by this method is very unstable, and its purification is still being investigated.

Aqueous extraction by the method of Best and Scott (27) resulted in a consistent yield of 75 units per kg. under the most favourable conditions.

Piper, Allen and Murlin's (28 29) aqueous extraction method gave yields of 100 to 200 rabbit units per kg. By the present writers' original method (30) yields of 230-400 rabbit units per kg. were consistently obtained. The introduction of later improvements to the original method increased the yield by aqueous extraction to about 1000 units. The introduction of the acetone-picrate method (Dodds and Dickens (31)) increased the average yield to about 2500 rabbit units. In one case, from pig-pancreas a yield of 4280 rabbit units per kg. was obtained. From ox-pancreas the average yield is also about 2000 rabbit units per kg. A number of other investigators have agreed that better yields are obtained from pig-pancreas. It has been suggested that this fact is connected with the animals' diet, but there is nothing to confirm this.

(*b*) *Fish-pancreas.*—The insulin content of the principal islets of the cod, halibut and other common fishes has been investigated by Macleod (88), McCormick and Noble (89), Dudley (90), and Swale Vincent, Dodds and Dickens (91).

This subject is of interest since in some of these fishes (the Teleostei) there is a large "principal islet", discovered by Rennie, which is often encapsulated, and thereby separated from the zymogenous tissue. Rennie (92) held the view that these principal islets are similar to the islets of Langerhans in the mammalian pancreas. If this is so, an opportunity occurs for ascertaining whether the islets of Langerhans or the zymogenous tissue are really the source of insulin in the pancreas, for it should be possible to prepare from the principal islets an extract

having the same effect as insulin on the carbohydrate metabolism.

With this object in view Macleod (88) prepared alcoholic extracts from the principal islets of a number of bony fishes. The sculpin (*Myoxocephalus octodecim-spinosus* and *scorpius*) and the angler fish (*Lophius piscatorius*) were among those studied. In both cases the results of Macleod showed that very potent extracts could be prepared from the principal islets, and that the mesenteric tissue, on the contrary, gave no insulin. Macleod pointed out that these results "afford strong direct evidence for the hypothesis that insulin, as its name implies, is derived from the insular and not the zymogenous tissue of the pancreas".

This work was done early in 1922, and the method of extraction then in use gave only poor yields of insulin. Collip (93), Best, Scott and Banting (94), Best, Smith and Scott (95), and others later demonstrated the presence of insulin in almost all tissue, and some experiments of the present writers in conjunction with S. L. Baker (96), which were being carried on at this time, led to the same conclusion.

Swale Vincent and the writers (91) therefore repeated the work of Macleod, using a recent method of preparation, which was known to give much better yields of insulin than the methods which Macleod had at his disposal. The result of this investigation was to provide further evidence that, as Macleod states, the source of insulin is, in fact, the islet tissue. Owing to the more efficient method of extraction, we were also able to demonstrate the presence of insulin, in very much smaller quantity, in the mesenteric tissue.

Macleod had suggested that the islets might furnish a convenient practical source of insulin, and this aspect of the subject has been investigated by McCormick and Noble (89) in America and by Dudley (90) in England in connection with the fisheries of the two countries. In his experiments Dudley made use of the direct application of a solution of picric acid to the original tissue, a method

which the present writers were independently investigating at the time. This method of extraction was very successful, and Dudley obtained the high yield of 13·12 original Toronto units per gram of wet islet tissue. McCormick and Noble (89) in one instance obtained a yield of 11·7 units per gram of islets from the pollack. Swale Vincent, Dodds and Dickens have also obtained very high yields of insulin from fish-islets.

(*c*) *The Insulin Content of other Tissues.*—As soon as Banting and Best had obtained insulin from the degenerated pancreas of the dog, they naturally investigated the effect of extracts made in the same way from other tissues. The results obtained by administration to diabetic dogs of the extracts of liver, spleen, thymus and thyroid were investigated. The results were negative or inconclusive, doubtless owing to the fact that the extraction method used was not sufficiently efficient to produce measurable quantities of insulin. Collip (93) and Best, Scott and Banting (94) have obtained insulin from the blood of normal animals.

Best, Smith and Scott (95) have recently repeated these observations and extended them. They have investigated the effect on the blood-sugar of normal rabbits, produced by extracts of thymus, submaxillary, thyroid, spleen, liver, brain and muscle tissue from the ox, and of extracts of liver, heart, muscle, skeletal muscle, blood and some other tissues from normal and diabetic dogs. The benzoic acid method of Moloney and Findlay (23) was used in the preparation of the extracts. The average of a large number of estimations indicates that the following yields are obtainable from normal and diabetic dogs :

	Normal.	Diabetic.	
Pancreas	7·2 units	. .	Per 100 g.
Blood	0·97 ,,	0·55 units	,, 100 c.c.
Liver	0·69 ,,	0·50 ,,	,, 100 g.
Muscle	0·56 ,,	0·94 ,,	,, 100 g.
Heart muscle . . .	0·90 ,,	0·50 ,,	,, 100 g.

Best, Smith and Scott point out the necessity for bearing in mind the difficulty of obtaining an accurate assay by the rabbit method of standardisation. It is interesting that the highest yield of insulin from muscle tissue was obtained from the muscle of the diabetic animal. After the administration of extract of the calf's thymus gland to a completely depancreatised dog, Best, Smith and Scott found that the liver contained 6 per cent. of glycogen. This experiment shows definitely that the active principle extracted was insulin, and not some other substance which resembles it only in lowering the blood-sugar. Ether anæsthesia lowers the insulin content of the tissues of normal or diabetic dogs.

These results do not confirm the findings of Ashby (97), who prepared extracts by the Fisher-Shaffer method from the kidney, spleen and muscle of normal and diabetic dogs. Ashby stated that insulin-like material disappeared from the tissues very shortly after pancreatectomy.

Baker, Dickens and Dodds (96) have pointed out that Ashby's extracts resemble the "glucokinin" described by Collip, and cause a preliminary rise of blood-sugar followed by a diminution which may last for as long as twenty-four hours. The work of Fisher (98) already referred to has shown that this effect may be due to the presence of two fractions, a toxic fraction which raises the blood-sugar and another which lowers the blood-sugar, and is what is generally known as insulin. This may account for the anomalous effects obtained by Ashby. In order to eliminate this fraction we applied the acetone-picrate method of extraction (33) to their estimation of the insulin in small quantities of tissue, and also by this means investigated the insulin content of various organs in the ox, pig, horse and sheep. The results are given in the table on following page.

Very little idea of the insulin distribution can be gained from these figures owing to the difficulty of obtaining all the organs of one animal. It can be seen, however, that the liver and salivary gland contain almost as much insulin *per gland* as the pancreas. The figures also show

Animal.	Organ.	Weight in Grams.	Rabbit Unit per Gland.	Rabbit Unit per Kilogram.
Ox	Pancreas	300	750	2500
	Salivary gland	50	710	35
	Ovary	..	..	Inactive
	Liver	3000	600	200
Pig	Pancreas	100	428	4280
	,,	78	294	3000
Horse	Pancreas	250	375	1500
Sheep	Pancreas	100	100	1000
Cat	Pancreas	1	2	2000

that the pancreas of the horse and sheep is suitable for commercial preparation.

(*d*) *Human Tissues.*—Since the diet of men differs very much from that of animals, it was thought that an investigation into the distribution of insulin throughout the human body would yield interesting results (Baker, Dickens and Dodds (96)). The difficulties in the way of such an investigation are very great—firstly, the question of freshness of tissues, and secondly, the difficulty in procuring more or less normal organs.

In the table on following page will be found the results of analysis performed on cadavers showing various lesions.

The results obtained from the analysis of human tissues show that insulin disappears very rapidly after death. Thus in one case examined eight hours after death no trace of insulin could be found.

The low insulin content obtained from the pancreas as compared with other organs, such as the kidney and spleen, might be explained by the fact that the pancreas alone contains an active proteolytic enzyme, which is known to destroy the activity of insulin. This action would presumably begin immediately after death. The high insulin content of the organs of a diabetic after dying in coma is again remarkable, and compares with Best, Smith and Scott's results with the insulin content of the muscle of normal and diabetic animals.

Age, Sex.	Cause of Death.	Number of Hours between Death and Analysis.	Pancreas.		Kidneys.		Brain.		Spleen.		Liver.		Muscle.	
			Per Kg.	Per Gland.	Per Kilo.	Per Gland.	Per Kilo.	Per Org.	Per Kilo.	Per Org.	Per Kg.	Per Org.	Per Kilo.	Per Org.
62, Male .	Pulmonary embolus	3	870	98·5	1166	365	..	..	900	164	250	410	..	..
60, Female	Sarcoma of neck	6	nil	nil	..	..	122	122	..	..	40	80	nil	nil
63, Female	Myocardial degeneration	2	1080	43	115	20·7	225	252	..	..	..	..	352	?
32, Male .	Basal meningitis	8	nil	..	nil	..	nil	..	nil	..	nil	..	nil	..
Fœtus .	Stillborn	1	380	5	..	..	..	..	..	..	..	..	..	..
43, Female	Diabetic coma	3	425	17	..	..	..	..	..	..	163	180	..	..
44, Female	Acute pancreatitis	8	470	?	40	..	..	..	..	..	40	..	..	..

(*e*) *Insulin from other Sources.*—In addition to being present in almost all the organs of the body of man and animals, insulin or insulin like substances occur in the vegetable world.

Collip (93) showed that an extract could be prepared from a variety of green vegetables which had the property of lowering the blood-sugar in normal rabbits, and in one case that of a diabetic dog. The effect of this extract takes place very slowly, and there is sometimes a preliminary rise of blood sugar. For this reason Collip was unwilling to name the substance "insulin", and devised the word "glucokinin" for extracts possessing this property. In view of the work of Fisher (98) already referred to, it is probable that Collip was dealing with a mixture of substances, one of which raised the blood-sugar, and the other lowered it. Some experiments performed by the authors on the preparation of insulin by a different process from potatoes and onion have shown that by the use of another extraction method no delayed effect on the blood-sugar is observed.

Best and Scott (99) have also prepared a substance, which has a rapid effect on the blood-sugar, by using Moloney and Findlay's benzoic acid method already described. Substances were prepared from rice, wheat, celery, beetroot and potatoes, which had a rapid and very marked effect in lowering the blood-sugar, and produced hypoglycæmic symptoms and convulsions in normal rabbits. The convulsions were relieved by administration of glucose, but paralysis of the hind legs of the test animals and their incomplete recovery indicated the presence of toxic substances in some of the cruder extracts.

These fractions appear to be eliminated if a process of preparation which gives a pure product is employed. The insulin-picrate process of Dodds and Dickens has been applied to this extraction and appears to eliminate the toxic fraction. The yields obtained were as follows :

Potatoes yield 60 rabbit units per kg.
Yeast yields nil.
Onion yields 70 rabbit units per kg.

An anti-diabetic hormone has been prepared from yeast by Winter and Smith (100) and by Collip (93). In addition to lowering the blood-sugar of normal rabbits, it was injected into persons suffering from diabetes mellitus with beneficial effects (Winter and Smith (101)). The alkaline extraction process of Dudley, already described, was employed in a later investigation by Hutchinson, Winter and Smith (102). Much trouble was experienced in finding a suitable kind of yeast. The same delayed action on the blood-sugar as Collip had previously obtained with extracts from other sources was observed.

Whilst working with the effect of fermentation on the activity of the hormone prepared from yeast, Winter and Smith were led to test the possibility of the production of the hormone by some micro-organism which is not a yeast itself, but is present in commercial yeast. They found that the pure culture of a bacillus from commercial yeast grew rapidly when sown into 200 c.c. of peptone water containing 1 per cent. of glucose and 1 per cent. of Na_2HPO_4, and incubated at 37° C. After two days an alcoholic extract was prepared and purified. The effect of this extract was to lower the blood-sugar of a normal rabbit from normal to 0·04 per cent. in seven hours, when the animal convulsed, and was revived by injection of glucose.

The Preparation of Solutions of Insulin for Clinical Use

The main points in the preparation of clinical insulin are :

(1) The potency must be uniform ;
(2) Stability of the product under all climatic conditions ;
(3) Sterility of the solution.

The tests for potency and the standardisation of solutions of insulin have already been dealt with. It is customary to use solutions containing 20 clinical units per c.c., but the actual potency has varied. Originally the clinical unit was one-third of the original Toronto

rabbit unit, that is, it was one third of the quantity of insulin required to lower the blood-sugar of a normal 2 kg. rabbit from a normal value of about 0·11 to 0·045 per cent. within four hours. Recently an increase of 40 per cent. upon this value has been announced. The strength of commercial preparations to day is probably rather more than this concentration. The solution is prepared in sterile distilled water, the pH of which is suitably adjusted. Since insulin is more stable in acid solutions, it is desirable that the reaction should be faintly acid; about pH 4·5 is a suitable acidity, and injection of a solution of this reaction is not painful.

Insulin solutions are usually put up in small phials similar to vaccine bottles, covered by an air-tight and sterilisable rubber cap, which is punctured by the needle when insulin is required to be withdrawn into the syringe for injection. It is of course important that the protein content should be low, or local irritation may be set up on injection. Generally speaking, it is inadvisable to use insulin for clinical use if it contains more than 0·5 mg. of solids per original Toronto rabbit unit. In the case of insulin of lower activity than this, further purification of the material is indicated before the preparation is suitable for clinical use.

The stability of the product is an important consideration, particularly when insulin is to be used under tropical conditions. This is best ascertained by incubating a previously carefully standardised sterile specimen, and withdrawing samples for testing on rabbits from time to time.

Solutions obtained from carefully prepared insulin in sterile distilled water containing a small percentage (about 0·25 per cent.) of tricresol are, in the authors' experience, usually sterile; but in all cases sterility tests are absolutely essential before the solution is used clinically, and filtration through a Berkefeld filter is very desirable.

If this is done it must be remembered that insulin is exceedingly readily adsorbed from solutions the pH of which is in or near the region 5-6, and the solution must be made acid to about pH 3 or alkaline to about pH 7

before filtration. The filter should be washed out with a suitable solution of the same pH before the filtration. To the filtered solution the tricresol or other preservative is added, and the solution then requires to be tested for sterility, care being taken to dilute the specimen sufficiently with a large volume of medium so as to eliminate the effect of the tricresol. Special care should be taken to incubate some specimens with anaerobic precautions, since the most dangerous organisms are likely to be found in this group, more particularly the spores of tetanus or anthrax and of other resistant organisms. If there is no growth within a sufficient period the batch of insulin may then be used for clinical purposes.

Administration of insulin by the mouth is impracticable on account of the destructive action of proteolytic enzymes, which destroy the activity of the hormone. Other methods of application which have been suggested have not been much used in practice. Telfer (103) has prepared ointments of crude insulin with lard or lanoline, which he rubbed into the skin of the abdomen of rabbits. Some of the insulin was absorbed into the blood, and in one rabbit convulsions resulted. In two other animals the blood-sugar was lowered considerably, but the quantities of insulin required to produce the effect were very much greater, approximately ten times more, than were required when injected subcutaneously.

Hachen and Mills (104) have found that insulin placed in the small intestines of rabbits and dogs is rapidly absorbed. They found that large doses of insulin given orally to normal and diabetic men had no effect on the blood or urinary sugar content, nor had the introduction of insulin directly into the duodenum.

Winter and Smith have given insulin, in solutions containing alcohol, by the mouth to rabbits, and in a few cases of diabetes in the human subject insulin has been given in the same way. The effect, however, is uncertain, and large doses are required, so that these methods cannot be regarded as practical substitutes for subcutaneous injection.

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CHAPTER II

THE INTERNAL SECRETIONS OF THE PITUITARY BODY (TETHELIN, PITUITRIN, HYPOPHYSINE)

Historical

THE functions of the pituitary body are so closely bound up with its anatomy that it is all but impossible to discuss them without a very brief reference to morphology. The gland consists of three parts, each differing in development, structure and function. The anterior lobe is originally developed as an outgrowth from the pharynx, the connecting stalk being finally absorbed. On microscopic section it is found to consist mainly of clusters of epithelial cells and connective tissue. Behind this lobe is a thin layer called the pars intermedia, consisting of ependymal and neuroglial cells. This connecting link joins the anterior lobe to the posterior lobe, which is a down growth of the brain. Unlike the stalk of the anterior lobe, that of the posterior persists to connect it with the brain. In structure it resembles the pars intermedia. An apology is needed for this elementary description of the body, but it is added for the benefit of non-medical readers in order that they might appreciate that, although extremely small, the gland consists of two different parts with different origins, and consequently it is not surprising that their functions may be quite separate.

Scientific interest was not aroused in the body until Marie (1) in 1886 called attention to the relation between acromegaly and diseases of the pituitary. In the same

year Horsley (2) endeavoured to reproduce the disease by removal of the gland, but it was not until Paulesco (3) had perfected the operative technique that any definite conclusions could be arrived at. He demonstrated conclusively that removal of the whole gland caused death. By a series of experiments he showed that only the anterior lobe is essential to life, whilst removal of the posterior lobe produces little or no symptoms. In 1909 Cushing (4) published the results of his researches. He proved that removal of the anterior lobe was followed by weakness and death. Polyuria and glycosuria resulted occasionally. Removal in older animals was more rapidly fatal than in younger ones, who occasionally became fat and sluggish before death. A series of clinical conditions gradually became associated with diseases of the pituitary body, and from this evidence it became obvious that the anterior lobe in some way controls growth, whilst the functions of the posterior lobe and pars intermedia remained obscure. For a full discussion of the pathology of the gland Harvey Cushing's book (5) should be consulted.

From 1905 onwards many papers were published on the effects of feeding animals either on the whole gland or on the separate lobes. Thompson and Johnson (6) noted a diminution of weight after feeding dogs upon sheep pituitary. They described various urinary changes, but these were not confirmed by Oswald (7). Franchini (8) studied the effect of injecting an aqueous extract of the body intravenously into rabbits. Various metabolic changes occurred, and some of the animals died. Caselli (9) gave subcutaneous injections of glycerol extracts without any effect on growth. Doubtful increases in weight and size were produced by Landri (10), who fed rats on dried glands.

Schafer (11) performed a series of careful experiments on feeding rats, and showed that an increase in the rate of growth occurred from the sixth to the twelfth week of life. He was the first to suggest that the age of the animal was of extreme importance in these experiments.

Aldrich (12) described a retardation of growth in animals fed on anterior lobe preparations.

In 1912 Lewis and Miller (13) conducted an elaborate series of feeding experiments, using anterior and posterior lobe separately. Comparing these animals with controls, they came to the conclusion that the experiments showed no effects whatever. Many of these observers noted that in animals fed upon whole glands and anterior lobes, prematurity and enlargement of the reproductive organs occurred. Posterior lobe administration is not attended by these changes, but increased intestinal peristalsis and diarrhœa are common.

Very little attention was paid to the chemistry of the gland until recent years, with the exception of various ultimate analyses. In 1904 Malcolm (14) published analyses of the lobes for phosphorus, nitrogen and calcium. Costelli (15) investigated the lipoids present, and Fenger (16) in 1915 extended these observations and analysed the ash of the body.

A further series of analyses were started by the discovery of hypertrophy of the pituitary in cases of athyroidism, since it was held that the pituitary might be able to carry out the functions of the thyroid in the absence of the latter. A series of investigations was commenced to find out whether the gland contained iodine. The presence of this element was found by some and denied by others. The question was finally decided in the negative for man by Denis (17) in 1911, and for sheep in 1920 by Seamen (18). Further investigations have shown that many conditions, such as pregnancy, can cause the pituitary to increase in size.

Anterior Lobe Extracts—Tethelin

Brailsford Robertson (19) in 1916 again called attention to the importance of the age of animals experimented upon, and pointed out that it would be only reasonable to expect that a young growing animal would respond differently from an old fully-grown one. He commenced

a series of experiments, and has stated that an active principle can be isolated from the anterior lobes. This he called tethelin.

Method of Preparation.—The method about to be described is taken from Brailsford Robertson's English patent, No. 15,683, 1915.

Fresh pituitary bodies of any animal are collected, dissected, and the anterior lobes are freed from the pars intermedia and posterior lobe. After thorough, fine mincing, the tissue is dried by mixing with three times its weight of a mixture of equal weights of anhydrous calcium and sodium sulphates. When an even paste is formed it is dried in a steam oven. If the mixture sets solid it is broken up and ground in a mortar until a uniform powder is obtained. This powder is thoroughly extracted with boiling anhydrous alcohol for some time, after which the solid matter is filtered off. The clear alcoholic liquid is then distilled under reduced pressure until solid just begins to separate out on cooling.

One to one and a half volumes of anhydrous ether are added, and the resulting precipitate is filtered off. The precipitate on the filter is washed with another volume of anhydrous ether, and the combined clear filtrates are dried rapidly at a low temperature in a perfectly dry atmosphere. The residue is the desired active principle —tethelin—of the anterior lobe.

Properties.—When obtained in a pure form it is a white or cream substance, and is readily powdered. It is very deliquescent, becoming a sticky, dark-coloured mass. When heated it begins to darken at a temperature of about 100° C., and the higher the temperature the darker it becomes. At high temperature it softens. On analysis the compound was found to contain 1·4 per cent. of phosphorus, and nitrogen in the proportion of four atoms to one of phosphorus. The material is soluble in water, alcohol, ether, chloroform and carbon tetrachloride. A mixture of dry alcohol and ether, in the proportions of one to one and a half respectively, fails to dissolve the compound. Employing Wig's solution, the iodine

absorption is stated to be 33 per cent. of its weight of iodine.

Reactions.—Prolonged treatment with water lowers the iodine value. The saponification value, expressed for 1 g. of substance, is 98 mg. of potassium hydroxide when phenolphthalein is used as an indicator. Treatment with sodium nitrite and acetic acid causes evolution of nitrogen. About half of the total nitrogen can be liberated in this manner. The addition of a saturated aqueous solution of barium hydroxide to a concentrated aqueous solution of tethelin causes a precipitate to form. This dissolves on heating, giving a yellow solution and a granular precipitate. After prolonged boiling, treatment with sodium nitrite and acetic acid liberates nitrogen to the extent of three-fourths of the total. Aqueous solutions show no power to reduce alkaline copper solutions. Millon's reaction is positive, but the pink colour disappears on the addition of more of the reagent. A positive Ehrlich's aldehyde reaction is given, even after prolonged boiling with barium hydroxide solution, but not if sulphuric acid is subsequently added to the boiled solution. The addition of chlorine, either before or after boiling with barium hydroxide, results in the production of a pink colour, which is discharged by the addition of excess of the reagent. If a solution of tethelin, after boiling with barium hydroxide, or with barium hydroxide followed by sulphuric acid, be saturated with chlorine and then boiled and evaporated to dryness, ammonia turns the deposit red on warming. If aqueous solutions, either alone or with barium hydroxide or sulphuric acid, be evaporated to dryness after the addition of nitric acid, a yellow residue results, the colour being deepened by treatment with ammonia. No red or violet colour results from heating tethelin with sulphuric acid. From a study of the decomposition products produced by boiling tethelin solutions with barium hydroxide the following have been identified :

1. An unsaturated fatty acid compound.

2. Inosite.
3. Possibly bodies allied to inosite.

From a detailed study of its reactions Brailsford Robertson concludes that it contains the following groups:

1. An iminazol group.
2. An unsaturated fatty acid group.
3. dl.-inosite.

Physiological Properties. The substance is non-toxic, whether administered subcutaneously or even in large doses, when given by the mouth. Intravenous injection causes a transient fall in the blood pressure, but does not give any of the characteristic physiological reactions of extracts obtained from the posterior lobe.

Schmidt and May (20) suggested that the presence of the iminazol nucleus might be accounted for by the fact that tethelin was in reality a combination of the secretions both of the anterior and of the posterior lobe. If this were true, it ought to be possible to split off a substance giving the typical reactions of the posterior lobe extract, which are not given by tethelin itself. Fission, by means of enzymes and bacteria, gave no positive results, so it was decided to examine the separation produced by the addition of barium hydroxide to the solutions of tethelin.

On adding barium hydroxide solution to an aqueous solution of tethelin an immediate, white, flocculent precipitate settles out. After filtration a clear yellow solution results, from which the excess barium can be precipitated by passing in carbon dioxide and subsequent filtration. A little barium bicarbonate remains in the solution. This is removed by concentration, when barium carbonate settles out. The resulting clear solution contains all the amino nitrogen of the tethelin. This solution, it is stated, gives all the physiological actions of pituitrin (extract of posterior lobe). The authors state that they have excluded the possibility of contamination with posterior lobe in the preparation of the original tethelin by a series of controls. Robertson (*loc. cit.*) claims that the substance is a definite chemical compound,

and that it stimulates growth. This growth-stimulating action was tested upon a number of white mice. A dose of 4 mg. per day was given orally to mice between the fourth and sixtieth weeks of age. During the first half of this period there was a marked retardation of growth, whilst in the later period there was a decided increase in the rate, as shown by weight. In all experiments a large number of animals were employed, and the statistical method of recording results was adopted.

Mice which had been previously starved for twenty-four hours recovered their loss of weight much more rapidly when tethelin was added to their food than did controls on the same diet with no tethelin. Mice fed on tethelin are said to be more compactly built than control animals, but are weight for weight smaller than the normals of the same age and size. Tethelin-fed males are more active than control animals. Robertson and Ray (21) performed an extensive series of feeding experiments, using whole gland, tethelin, egg lecithin and cholesterol. A large number of control experiments were performed.

The following conclusions were arrived at :

1. Errors in predicting the rate of growth of rats are greatest during the first thirty weeks, when the growth may be most irregular.
2. After the thirtieth week the error diminishes progressively, showing that all animals tend to approach a final condition of equilibrium, and that the variability in different animals consists in the time taken to reach this state of equilibrium.
3. These remarks are true of animals receiving any of the four test substances.
4. The tethelin-fed animals reached this stage of growth equilibrium much earlier than the others. Cholesterol also showed some activity.
5. With the exception of the tethelin-fed animals, the effect on females was much greater than on males during the first thirty weeks. With tethelin the reverse is true.

6. In later weeks females may show much greater growth than males.
7. As already stated, tethelin affects the early rate of growth, but does not affect the animal when a stage of equilibrium has been reached.

In a later paper it was shown that tethelin feeding increases the duration of life by 13 per cent. in males and by 11 per cent. in females. Animals killed after prolonged administration of the substance show very frequently carcinomatous changes in the viscera. Robertson stated that the mortality from carcinoma in tethelin-fed rats is much greater than in control animals.

Administration of small amounts of tethelin for a short period was found to be followed by a retardation in the growth rate, which, after a few days, was replaced by an increase. Robertson and Ray (*loc. cit.*) conclude that the retardation is due to tethelin, whilst the acceleration is due to the animal's own response.

Robertson and Ray also noted that tethelin stimulates the healing of wounds, and also greatly accelerates the rate of growth of inoculated tumours in animals.

Schmidt (22) proved that the substance possessed no antigenic properties whatsoever, and that it had no action on the growth of various bacteria. Corper (23) investigated the effect of tethelin upon the progress of tuberculosis, and came to the conclusion that no arrest of the condition took place. Ewe (24) has described various pharmaceutical points with regard to the preparation of tethelin solution for clinical use. Various incompatibilities, such as iodine and oxidising agents, are noted, and the author suggests that tethelin should not be stored except in the dry condition.

Robertson and Burnett (25), and also Barney (26), have discussed the question as to which particular groups in tethelin are responsible for its action. The former consider that the presence of unsaturated groups with the formation of hydroxyl groups may account for its properties. The presence of a hydroxylated benzene group

alone would not account for its activities, since various other substances, such as borneol and inositol, are inactive. Barney states that in addition to tethelin, cholesterol and lecithin are active, and suggests that the activity of the three substances must be due to some common group.

Drummond and Cannan's Criticism of Tethelin.—The literature has been searched very carefully for publications dealing with tethelin other than those of Brailsford Robertson's school. One paper has been found, and that is by Drummond and Cannan (27), published in 1923. They entirely failed to support Robertson's claims, and the paper is rather a devastating criticism of his views. Thus Drummond and Cannan have proved that tethelin is not a pure substance, but consists of a mixture of lipoids, and therefore is impure. They also performed a series of experiments on the growth-controlling powers of the material, and were unable to demonstrate any action whatsoever. In fact, they were unable to confirm any of the statements made by Robertson and his co-workers. Robertson (28) replied to this in 1923, and stated that the failure of Drummond and Cannan's experiments was due in the first place to their employing a faulty method of preparation, and in the second to the fact that they did not use the statistical method of comparing the growth rate in tethelin-fed rats and in normal controls. It is to be hoped that more experiments upon these lines will settle definitely the claims of tethelin.

Evans and Long, in their memoir to the University of California (1922, vi.), describe experiments upon the effect of administering fresh anterior-lobe extracts *via* the peritoneal route. They obtained almost giant rats; for instance, at the end of 333 days' treatment one of the injected rats weighed 596 g., as compared with 248 g. for the control of the same litter. They state that the extract is inactive if given by the mouth.

The Internal Secretion of the Posterior Lobe

Earlier investigators devoted the main part of their time to studying either the functions of the whole gland or those of the anterior lobe.

The various feeding experiments have already been dealt with, and it remains to consider a few historical points concerning the functions of the posterior lobe. Although scientific interest was first aroused in the gland by Marie (1) in 1886, it was not until 1895 that Oliver and Schafer (29) published an account of the effect of injecting extracts of the posterior lobe. They demonstrated a pressor effect, which was afterwards confirmed by Howell (30), 1898, Schafer and Vincent (31), 1899, and Magnus and Schafer (32), 1901–1902. These publications directed the attention of physiologists to the properties of the posterior lobe, but it remained for Dale (33) in 1906 to add the most important stimulus for investigation. He demonstrated the effect of pituitrin upon the excised uterus. From this date many papers appeared, reporting methods for the preparation and study of this extract.

Method of Preparation.—Practically all the original experiments upon the physiological properties of the posterior lobe of the pituitary were made with saline extracts. Since the greater part of the researches upon this preparation have been carried out by commercial firms, the majority of our information is derived from patent sources. One of the first methods evolved was patented by Meister, Lucius and Brüning. Our account is taken from the D.R.P. 264,119 (21.II.1913).

The posterior lobes are dissected out, and after thorough mincing the tissue is extracted with weakly acidified water. A relatively large volume of this dilute acid is employed in order to be sure of thorough extraction. Albumin and allied substances are removed by dialysis, and the resulting solution is evaporated *in vacuo* to a small volume. A fairly strong solution of some salt of a heavy metal, such as silver, gold, platinum or mercury, is added. This causes the appearance of

a precipitate, which is filtered off and washed. The metallic portion of the precipitate is then removed "in the usual manner", probably by solution in acid followed by precipitation with hydrogen sulphide. The solution of the metal-free precipitate may be used clinically, or the solid may be obtained on evaporation *in vacuo*. The resulting substance possesses all the properties of a saline extract of the posterior lobe. Although the details are somewhat meagre, the general outline of the process can be appreciated. Discussion of the chemical properties will be postponed until all the methods of preparation have been described.

The same firm obtained another patent in 1912 (D.R.P. 268,841, 4.IV.1912). Here they described a method for preparing the active principle in a crystalline form. The acid dialysate described previously is treated with any of the usual alkaloidal precipitants, such as phosphotungstic, tannic or picric acids, or iodine in potassium iodide. Salts of heavy metals must not be used. The precipitate is filtered off, washed and freed from the precipitant by some suitable means. The resulting solution is then evaporated and allowed to crystallise. This crystalline substance is said to possess all the pharmacological properties of the posterior lobe.

Fühner's Hypophysine.—In 1913 Hermann Fühner (34) published an account of a method. The finely minced posterior lobes are extracted with water, and the resulting solution is freed from proteins, presumably by dialysis. Any non-metallic alkaloidal precipitant, such as phosphotungstic acid, is added, and the resulting precipitate is filtered off and washed. The compound is then decomposed by baryta, the excess being removed by the addition of sulphuric acid. After filtration the clear liquid is evaporated *in vacuo*, and allowed to crystallise. The resulting product is composed of pale yellow crystals, the sulphate of the active principle. Fühner called this body hypophysine. It is soluble in weakly acid or alkaline water, soluble with difficulty in acetone, alcohol and acetic ether. The aqueous solution is lævo-rotatory,

and gives the Pauly reaction (red colour with diazotised sulphanilic acid) (see p. 94). With sodium hydroxide amino bases are split off in the cold. The crystals give the biuret reaction.

Working with the original protein-free extract of the posterior lobes, Führer claims to have isolated eight separate fractions, four acting upon the uterus and being present in hypophysine, and four more bodies from the phosphotungstic acid precipitate. The physiological properties will be discussed later.

In 1913 Hoffman la Roche Company obtained a patent for an alkaline method of extraction (D.R.P. 282,002, 30.I.1913). The posterior lobes are ground up with sodium carbonate, and the resulting powder is extracted first with chloroform and then with alcohol. These extracts, after solidifying, are evaporated to dryness, and it is stated that the resulting powders are active. A further patent by this firm describes another method (D.R.P. 284,148, 5.II.1915). They state that clinical and experimental evidence points to the fact that the active principle is soluble in absolute alcohol, and consequently they recommend extraction of the dry crude pituitrin with this solvent. By this means inert material, such as amino acids and proteins, is eliminated. If anhydrous ether be added to the alcoholic solution after the addition of hydrochloric acid, pituitrin chloride separates out in crystalline form. If this process be repeated several times, highly active material can be obtained. If sulphuric acid is added in place of hydrochloric the sulphate separates out. The product is soluble in water and in alcohol, and with mineral acids hygroscopic salts may be obtained. The nitrogen content is said to be about 10·8 per cent.

Aldrich in 1918 described a method consisting of extracting the defatted and dehydrated posterior lobes with glycerol. The active principle could be precipitated from this solution by the addition of acetone (U.S. Patent 1,271,111, 2.VII.1918).

Flaecher and Reuter (U.S. Patent 1,296,064, 4.III.1919)

described a process almost identical with that of Fühner (*loc. cit.*). An acid, aqueous extract is freed from albumin and is concentrated by evaporation. Sulphuric acid and phosphotungstic acid are then added, and the precipitate formed is filtered off and washed with very dilute sulphuric acid. It is then ground up to a thin paste with barium hydroxide until a definitely alkaline reaction is obtained. The barium phosphotungstate is then filtered off, and any excess of barium is precipitated by the addition of sulphuric acid. The liquid is concentrated *in vacuo* and allowed to crystallise ; or the base may be precipitated by the addition of acetone or alcohol. The properties were the same as those of Fühner's compound.

During the year 1919 a series of very important observations was published. Abel and Kubota (35) obtained β-iminazolyl ethylamine from pituitary tissue, and concluded that this substance was responsible for the oxytocic action of the extracts. Although this work will be discussed in detail later, it is mentioned here in relation to the work of Dudley (36) who disproved their theory. Dudley published a very full account of a method for the preparation of the uterine-contracting substance.

Dried, powdered posterior lobe tissue is extracted with one hundred times its weight of faintly acidified hot water (two to three drops of 2 N acetic acid per 10 c.c. of water). After heating for ten minutes and filtering, a perfectly clear solution is obtained, containing about 20 per cent. of the weight of the original powder. This is purified by the addition of colloidal iron. Five c.c. of the filtrate are titrated with a solution of colloidal ferric hydroxide. If this be added, drop by drop from a burette, it will be seen that at first a precipitate is formed, which sinks rapidly to the bottom, leaving the supernatant fluid clear. When one drop in excess has been added, the supernatant fluid assumes the brown tint of the ferric hydroxide solution. If a 5 per cent. solution of ferric hydroxide be employed, Dudley states that about 0·2 c.c. of colloidal iron is sufficient for complete precipitation.

When this figure has been arrived at, the titrated solution is poured back into the main bulk of solution, and the requisite quantity of colloidal iron solution to cause complete precipitation is added. The solution is then heated for two minutes in a boiling water bath, cooled and filtered. A clear, colourless liquid results. The next step consists in extraction of the aqueous solution with butyl alcohol at reduced pressure according to the suggestion by Dakin.

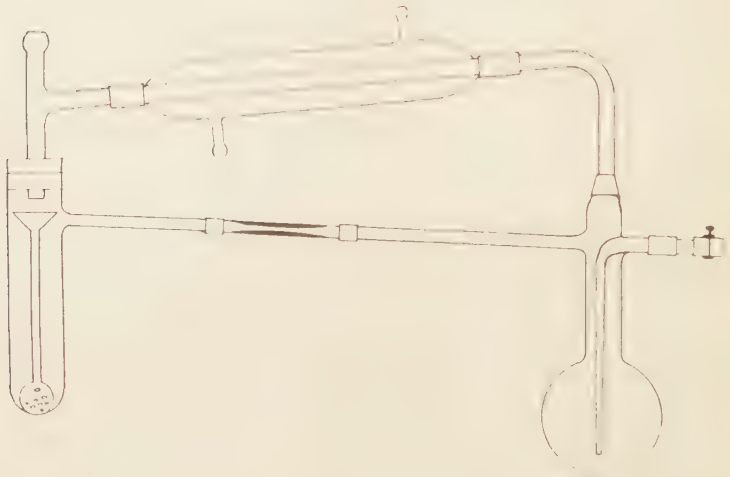


Fig. 2.—Apparatus for continuous extraction with butyl alcohol under reduced pressure. (Copied from Dudley's paper, *J. Pharm. and Exp. Therap.*, 1919, xiv. 295.)

The apparatus required (see Fig. 2) consists of a flask, fitted with a bubbler and a side tube set at right angles to the neck of the flask. The neck is fitted with a delivery tube leading to a Liebig condenser, sloping downwards, and connected with a wide glass test-tube by means of a T-piece. One end of this T-piece enters the test-tube, whilst the other is used for evacuation. The end entering the test-tube is placed immediately above another smaller piece of glass tubing, the end of which consists of a perforated bulb resting on the bottom of the

containing test-tube. The arrangement is such that any liquid condensed in the Liebig condenser will run through the T-piece into the test-tube, and will drop down into this perforated bulb.

The test-tube is also provided with a side tube inclined slightly downwards. This is connected by means of a constricted rubber junction with the side arm of the flask already mentioned. Butyl alcohol is placed in the flask, whilst the aqueous extract is put into the test-tube. The pressure is then reduced to about 10 to 15 mm., and the flask containing the butyl alcohol is heated in a water bath at about 45 to 50° C. The vapour passes up into the delivery tube, and is condensed in the Liebig condenser. The liquid then runs into the test-tube, down into the inner tube, and through the perforation in the bulb. The butyl alcohol rises to the surface of the aqueous extract, and collects there until it overflows back into the flask through the side arm. The constricted junction, by holding up the butyl alcohol, prevents its vapour from passing direct to the flask. By this means a process of continuous extraction is obtained. The process is allowed to proceed for from twelve to fifteen hours. The butyl alcohol extract is then evaporated and allowed to crystallise. By this means a highly active substance is obtained. It is contaminated with amino-acids and other substances extracted by the butyl alcohol. Dudley found that about 60 per cent. of the solids of the aqueous solution were thus extracted, and that there was no loss in activity.

In 1920 Crawford (37) described a process for the preparation of the pressor substance present in the posterior lobe of the pituitary. The tissue is extracted with either acid ethyl alcohol or 0·3 per cent. acetic acid. In the former case the active substance is precipitated with ether, whilst in the latter it is first brought down as a mercury compound. This is dissolved in acid, and the mercury is removed by passing a stream of sulphuretted hydrogen through the solution. After filtration this solution is evaporated to dryness *in vacuo*, and the

residual solid is extracted with acid alcohol, and the pressor substance precipitated by the addition of ether.

Clover obtained a patent for a process depending upon the salting out of the active substance (U.S. Patent 1,373,551, 5.IV.1921). The posterior lobe tissue is extracted with hot acidulated water, and after filtration the solution is saturated with common salt. A precipitate occurs, which is said to possess all the properties of pituitrin.

It is feared that the account of methods for preparation of an active pituitary extract are rather unsatisfactory. This is due to the fact that the only information available was in the patents of England, Germany and America. A very long and careful search through the literature showed that Dudley's paper (*loc. cit.*) alone gave full working details of a process, and in every other description the phrases "by some suitable means" or "after removing inert substances" were encountered.

We now come to a consideration of the chemical and physiological properties of this substance. Since there is no uniform process of manufacture, and since each observer has studied the properties of his own particular preparation without reference to those of others, it will perhaps be as well to describe the physiological reaction of extracts in general before chemical particulars.

Physiological Actions

(*a*) *Circulatory System.*—As already indicated, Oliver and Schafer in 1895 described a pressor effect of extracts. The rise in blood pressure is prolonged, and is not affected by destruction of either brain or cord. They concluded that the action of the extract was direct upon the musculature of the peripheral arterioles. This contention was also supported by the fact that the output of a perfused frog's heart and the splenic volume could be decreased under the influence of the extract. The intestinal muscles also contracted. Oliver and Schafer employed extracts of whole gland, and it was not until Howell, 1898 (*loc. cit.*),

repeated their experiments, using only posterior lobes, that the production of the active principle was definitely allocated to this pars posterior. Howell extended the earlier observations, and showed that the slowing of the heart was partially due to vagus action, since section of these nerves or treatment with atropine modified the effect. The pressor effect of a single injection may last twenty to thirty minutes, or even longer, but the injection of a further quantity of extract causes a fall in the pressure.

Schafer and Vincent (*loc. cit.*) demonstrated the presence of both a pressor and a depressor substance in saline extracts of the posterior lobes of ox pituitary, and stated that the former was insoluble in alcohol or ether. The active substances were not destroyed by boiling and could be dialysed. They also demonstrated very clearly the strong depressor effect of a second dose of extract, and suggested that it was due to the action of a depressor substance, which was not choline. Very little has been added to the knowledge of the effect of pituitrin upon the circulation since these early experiments. They have received abundant confirmation. Many observers (Mummery and Symes (38), 1908, Bell and Hick (39), 1909) have pointed out the difference between the pressor effect of pituitrin and adrenaline. Hoskins and McPeek, (40), 1913 14, showed that the pressor effect was unaltered after section of the adrenal vein. Fröhlich and Pick (41), 1913, and Hallion (42), 1914, suggested that vasodilatation might occur in certain parts of the circulation. Claude and Porak (43) in 1913 suggested that pituitary extracts had a selective toxic action upon heart muscle. This theory has received considerable support from various workers (Werschinin (44), Tigerstedt and Airila (45), and Wiggers (46)), who maintain that the secondary fall in blood pressure after two injections may be due to this action. Etienne and Parisot (47) found that repeated injections of pituitary extracts lead to cardiac hypertrophy.

Halliburton, Candler and Sikes (48) confirmed the

greater part of these reactions for extracts obtained from human posterior lobes.

(*b*) *Effect on the Kidneys and Secretion of Urine.*— Magnus and Schafer (*loc. cit.*) found an increased renal activity, as shown by an increased output of urine following the injection of extracts. These results have been confirmed by a number of workers (Schafer and Herring (49), Thaon (50), King and Stoland (51), and Hoskins and Means (52)), but have also been contradicted by other investigators (Pentimalli and Quercia (53), Garnier and Schulman (54), von Meyenberg (55), etc.). In fact the latter group of workers observed a decrease in the secretion of urine following injections of posterior lobe extracts. Magnus and Schafer (*loc. cit.*) observed a preliminary contraction, followed by a dilatation of the renal arterioles under the influence of pituitrin. They attributed the increased secretion both to increase in blood pressure and to direct stimulation of the renal epithelium.

Schafer and Herring (*loc. cit.*) in 1906 inclined more to the latter view. Knowlton and Silverman (56), however, in 1918 failed to find any increase in the renal oxygen consumption during the diuresis, and came to the conclusion that the effect was due to vascular changes. Addis, Shevky and Bevier (57), in the same year, noted a reduction in the rate of excretion of urea after the injection of pituitrin.

(*c*) *Effect on Lactation and other Secretory Processes.*— Ott and Scott (58) in 1910 were the first to demonstrate that pituitary injections increase the secretory powers of the lactating mammary gland. This work has received abundant confirmation (Mackenzie (59), Schafer and Mackenzie (60), Houssay and Maag (61)). Hofstätter (62) claimed to have produced hypertrophy of the gland by means of prolonged administration, but this reaction could not be obtained in the virgin animal (Bell (63), 1919). With regard to the mechanism of this process there is considerable doubt. Hammond (64) regarded the process in the light of stimulation of the secretory cells, since it had been stated that there was an increase in the

fat content under the influence of pituitrin. McCanlish (65) denied this in 1918.

Schafer (66) holds the view that the action of the hormone can be explained by a contraction of the plain muscles squeezing out the contents of the alveoli. Various other investigators support this theory (Gaines (67), Bell (*loc. cit.*)).

The effect of pituitrin upon the secretions of other glands is mainly inhibitory. Thus Rogers and co-workers (68) demonstrated an inhibition of gastric secretion, a fact afterwards confirmed by Pal (69). Wiggers (46) showed a similar phenomenon for pancreatic secretion, whilst Solem and Lommen (70) obtained a decrease in salivary flow after administration of the substance.

(*d*) *Effect upon Respiration.*—The greatest confusion exists in the views upon this subject. Investigations have been carried out along two main lines: the first is whether pituitrin increases or diminishes the amplitude of respiratory movement, and the second is whether bronchial constriction occurs. With regard to the first question, Mummery and Sykes (38), 1908, recorded a diminution in respiratory movements, whilst Houghton and Merrill (71), in the same year, found exactly the opposite. Nice, Rock and Courtright (72) attempted to harmonise these discordant views by suggesting that at first an increase is produced, which later is replaced by a decrease. A second injection produces no effect. There is no definite ruling upon the second question, Fröhlich and Pick (41) recording spasm and Trendelenburg (73) denying this action.

Later Fröhlich (74) maintained that pituitrin acted upon the vagal terminations in the bronchi. De Bomis and Susanna (75) recorded a constriction of the pulmonary arteries by the hormone.

(*e*) *Effect upon the Uterus.*—While conducting some experiments upon the action of ergot, Dale (33) noted that intravenous injection of pituitary extracts caused a marked contraction of the cat's uterus. There can be no doubt that this discovery in 1906 must be ranked

amongst the most important observations in this field, since it introduced pituitrin into clinical medicine and surgery. In a later publication Dale (33) showed that all plain muscle was affected similarly, and that the active principle was excreted in the urine.

These observations were confirmed by all workers, and the phenomenon is now used as the basis of a method of assay. The action is the same upon the pregnant or non-pregnant organ. Cow (76) recorded a relaxation under certain conditions. Full descriptions of these reactions will be considered under the headings of methods of assay and constitution.

(*f*) *Effects upon Metabolism.*—The results of various feeding experiments upon the metabolism of animals have been dealt with in an earlier section. Malcolm (77) in 1904 maintained that the hormone had a "catabolic" effect upon the metabolism of bone, resulting in an increase of fragility. In support of this Franchini (78) produced evidence that the calcium and magnesium content of the blood showed a decrease following injections. Frey and Kumpiess (79) recorded marked phosphaturia, whilst Farini and Ceccaroni (80) demonstrated an increased excretion of hippuric acid.

Cushing (81) described the appearance of glycosuria following operative interference with the gland. Goetsch, Cushing and Jacobson (82) found that injections of large amounts of posterior lobe extracts caused glycosuria, and later Cushing (*loc. cit.*) suggested that the action was due to glycogenolysis. Achard, Ribot and Binet (83) were able to produce hyperglycæmia by similar methods. Kojima (84) had previously shown that prolonged feeding with pituitary tissue gave rise to an œdematous condition of the pancreas. The zymogenous tissue showed marked vacuolation, and the islets were crammed with granules. The observation of Burn (85) upon the interaction between insulin and pituitrin has been described earlier.

The relation between the pituitary gland and the disease known as diabetes insipidus is fully considered in a paper by Kennaway and Mottram (86). Their general

conclusions, based upon observations made upon two cases, may be summarised as follows :

1. From analyses of blood serum and urine under resting conditions, and after the administration of sodium chloride, they demonstrated that the kidney had lost its concentrating power.

2. The injection of pituitrin controlled the polyuria in these cases, but no effect was observed when the substance was given by the mouth.

3. The anti-diuretic action of pituitary extracts appears to be due to direct action upon the kidney.

4. Since injections of pituitrin restore the urine to its normal condition, these observers concluded that the gland controlled the secretion of urine in some way.

5. They state that direct evidence (post-mortem, etc.) of the location of this function to the posterior lobe is inconclusive. A very fine bibliography is given at the end of their paper.

Mode of Absorption of Pituitary Principles

In conclusion, a few words must be said about the significance of colloid, and about the mode of absorption of the pituitary principle. The deeper portions of the cells bordering the interglandular cleft show a tendency to be arranged in small vesicles bearing a certain superficial resemblance to those of the thyroid gland. The vesicles often contain a hyaline or colloid material, which stains intensely with eosin. Opinions differ concerning the interpretation which should be placed upon this colloidal material. Some think it is a degenerative product, whilst others consider it to be a remnant of a hypothetical external secretion. By far the majority of recent investigators, however, look upon it as a true internal secretion. The increase in amount following removal of the thyroid or the pancreas supports this conclusion.

Herring has reported the observation of all stages in

the passage of the material through the posterior lobe into the cavity of the third ventricle.

Cushing and Goetsch (87) claim that the active principle of the posterior lobe is discharged into the cerebro-spinal fluid. This is contradicted by Carlson and Martin (88), whose criticisms appear to be accepted by a number of workers.

It now remains to consider the possible chemical constitution and methods of assaying the active principle. As the greater part of the work upon the former rests upon the method of standardisation it will be as well to describe this first.

Methods of Standardisation

As already pointed out, Dale (*loc. cit.*) was the first to demonstrate the uterine contracting, or oxytocic power of infundibular extracts. By means of a series of researches he was able to prove that the response was quantitative, and to develop this reaction into a method of assay. In brief, the method consists in the setting up of a strip of uterus in a suitable recording apparatus, and comparing the oxytocic action of the unknown with that of a standard solution.

These are the essentials of the method evolved by Dale and Laidlaw (89). The original technique has been modified in minor details by various observers, but the following account embraces most of the generally accepted views. It will be as well to consider the method under two headings –selection of the muscles and fixing in the apparatus, and the choice of a standard.

1. *Selection of Muscle and setting up Apparatus.*— Practically all workers prefer to use one horn of a virgin guinea-pig's uterus. Smith and McClosky (90), in their exhaustive account of the method, state that a virgin guinea-pig, whose weight lies between 180 and 250 g., is preferable. The most uniform results are obtained from young animals, preferably between the ages of three and five weeks, since the uterus will not behave irregularly at this age. After killing the animal by a blow on the

head, both horns of the uterus are removed and placed in Locke's solution. They are then freed from connective tissue and Fallopian tube, and one is set up in the apparatus (see Fig. 3), which consists of a glass vessel containing Locke's solution maintained at a constant temperature of 38° C. by some suitable device (such as an electric lamp or heater). The glass vessel, which may be jacketed if preferred, is provided with an exit at the bottom, so that the solution can be changed without disturbing the preparation. The isolated horn is suspended between two platinum hooks, one being attached to some fixed object, whilst the other is connected with a recording lever, arranged to magnify the changes about four times. If necessary the lever can be weighted. The level of the Locke's solution is so arranged that the preparation is completely submerged. Smith and McClosky recommend the following formula for Locke's solution :

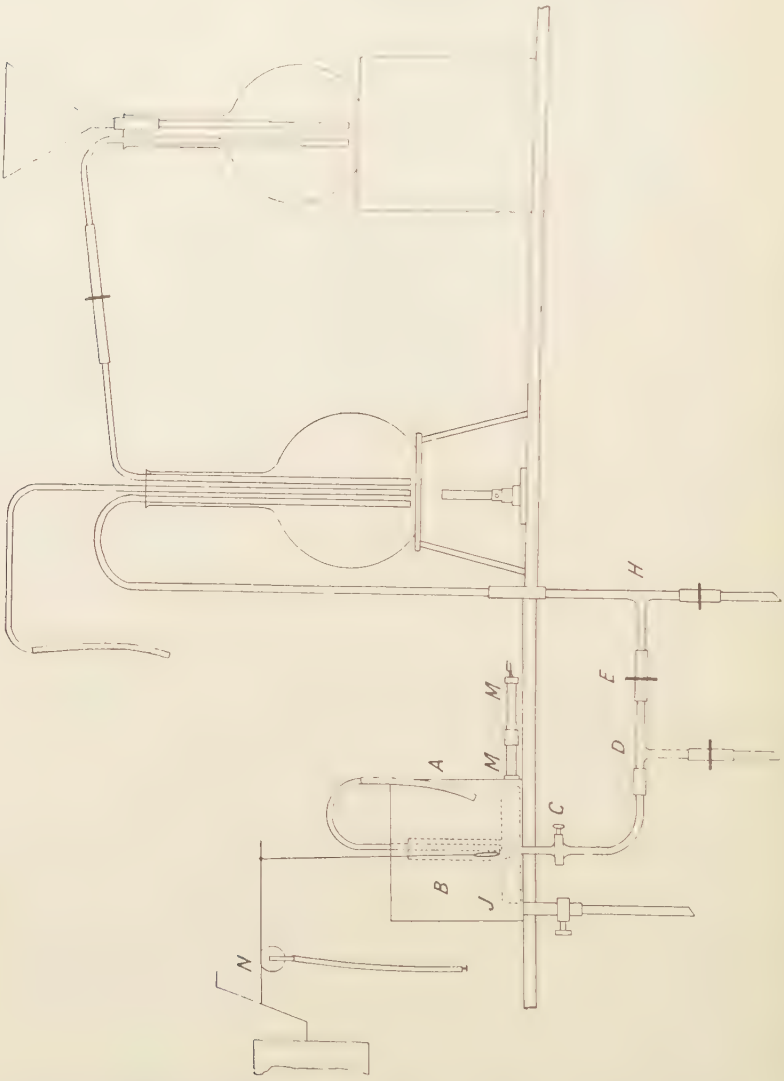
Sodium chloride	9·0 g.
Calcium chloride	0·24 g.
Potassium chloride . . .	0·42 g.
Sodium bicarbonate . . .	0·50 g.
Dextrose	0·50 g.
Glass-distilled water to 1000 c.c.	

Ringer's solution is employed by many workers.

Burn and Dale recommend the addition of magnesium chloride (0·0005 per cent.) to the above, since they state that by this means the tendency to irregularity is reduced.

Oxygen is bubbled through the solution, and Dale and Laidlaw (*loc. cit.*) recommend that the lower hook to which the uterus is attached should be situated upon the oxygen entry tube just above the end. Smith and McClosky (*loc. cit.*) found that much more uniform results were obtained if the oxygen were first bubbled through 500 c.c. of a 2 per cent. solution of sodium bicarbonate. A little carbon dioxide is carried over, which results in great stability of the preparation. The preparation is then left to itself for a short while, with the recording lever writing upon a revolving drum.

In a short while a constant base line will be obtained, with only occasional small spontaneous contractions.



The standard solution is then diluted with Locke's solution, and a small volume (0·5 c.c. if the volume in the

bath is 100 c.c.) is added, and the resulting contraction is recorded.

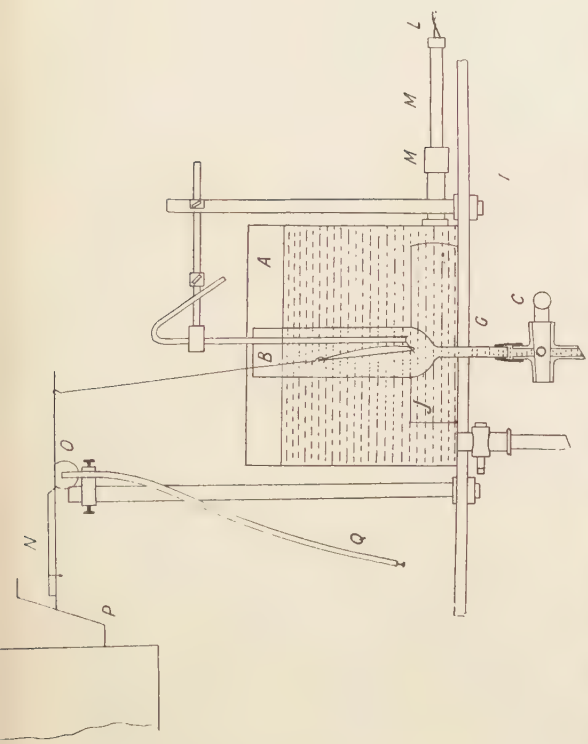


FIG. 3. Apparatus recommended by Burn and Dale for the standardisation of pituitrin. (Reproduced from the Medical Research Council's Special Report, No. 69, 1922.)

The uterine slip is set up as shown, in the glass vessel (*B*), which should have a cross-sectional area of about 9·5 sq. cm., and the exit tap (*C*) is connected as shown. The vessel is filled to a depth of 10 to 11 cm., requiring about 100 c.c. of solution to effect this. By means of the connections to the two large flasks, the chamber can be washed out with saline without removal from the thermostat bath (*A*). This is cylindrical, and should be about 18 cm. in diameter and height. The temperature is regulated by means of an electric lamp (*K*), which passes into the bath by means of a blind tunnel brazed into the side of the bath. The temperature can be maintained at a constant level by sliding the lamp in and out of the tunnel. The uterine slip is attached to the end of the oxygen delivery tube, and to the recording lever in the manner shown. The lever itself is made of thin magnalium, and is bent according to the description of Lovatt Evans. The lever can be braked by the Bowden wire arrangement (*Q*) so that it can be stabilised when changing solutions. Burn and Dale suggest 19 cm. as a suitable length, with the fulcrum 8·5 cm. from the end attached to the preparation. The magnification will therefore be in the proportions of 5:4. If necessary the lever may be weighted.

The solution is then changed, and the observation is repeated with the unknown preparation. Dilutions are

then made until the effect of the standard is reproduced. Then both standard and unknown are increased by 10 or 20 per cent., and confirmatory readings are taken. The diagram of the apparatus used by Burn and Dale (pp. 88, 89) renders the description clear.

II. *Choice of Standard.*—A great deal of controversy has been waged over this point. In their original work published in 1912, Dale and Laidlaw (*loc. cit.*) recommended the use of a preparation of the posterior lobes made under definite standard conditions. Since the preparation of standards of constant strength depends on the glands in addition to the method of extraction, many observers maintained that a natural standard was an impossibility. Roth (91) suggested the use of histamine, a chemically pure substance, in place of the natural product. The action of this substance upon the uterus is stated to be identical with that of pituitrin, and Roth employed a standard of pure β-iminazolylethylamine hydrochloride in water, 100 mg. per 100 c.c., *i.e.* a 1 in 1000 solution. The solution is then put up in ampoules of about 1·3 c.c. capacity, and is sterilised at 100° C. for twenty minutes on three successive days. After sealing, the phials are stored in a cold, dark room, and, after six months, are said to show no change. For each test a 1 in 20,000,000 dilution is required. For the actual test, 1 c.c. of the contents of the phial is diluted to 100 c.c. and 5 c.c. of this are diluted to a litre with Locke's solution. This gives the required final solution.

This method has been vigorously criticised by many workers. Based upon the use of an artificial standard, it is obviously open to many objections. Thus pituitary extracts contain other substance besides the oxytocic principle, which might influence the contraction. Also, the action of the pituitrin differs in some respects from that of histamine, which may undergo deterioration and alteration if not sterile.

By far the most important criticism is that offered by Burn and Dale, who proved that uteri differ in their sensitiveness to pituitrin and to histamine. This in

itself is sufficient to condemn the method. Moreover, they showed that "the sensitiveness of the uterus to histamine and to pituitary extracts changes in opposite directions", and therefore histamine is unsuitable as a standard. Later Spaeth (92) and Tate (93) suggested the use of a 15 per cent. solution of potassium chloride as a standard. Addition of such a solution causes a similar contraction to that of pituitrin. They pointed out that natural standards were unsatisfactory in the tropics, owing to autolytic changes, but that the adoption of potassium chloride as a standard would overcome this.

Practically all workers have returned to the original form of natural standard, as recommended by Dale and Laidlaw (*loc. cit.*) in 1912. A full account of the arguments concerning the adoption of standards will be found in Burn and Dale's communication in 1922 (94). They recommend the use of a 10 per cent. extract of fresh posterior lobes, and that all commercial preparations should be brought up to this strength.

Dale and Laidlaw originally prepared their standard by extracting the tissue with sufficient boiling acidulated water to make a 10 or 20 per cent. extract of the fresh moist gland. After filtration the solution is put up into small phials and is autoclaved.

In their recent publication, 1922, Burn and Dale (*loc. cit.*) make their extracts up to 2·5 per cent. of infundibular material, taking great care to employ absolutely fresh material.

Smith and McClosky (*loc. cit.*) prepare a standard powder, from which the standard solution can be made. The glands are obtained within twenty minutes of the death of the animal, and the posterior lobes are dissected out. They are immediately dropped into acetone, which dehydrates the tissue and stops enzyme action. The acetone is decanted after a short time, and the tissue is cut up into small pieces, and, after the addition of more acetone, it is stored in an ice-box for some time. By this means the glands are dehydrated and defatted, and, on removing from the acetone and drying in a vacuum-

desiccator, a fine powder may be obtained on grinding in a mortar. The powder is then passed through a No. 40 sieve, and is extracted in a Soxhlet apparatus for three hours with acetone. The material is then vacuum dried to a constant weight.

The powder is stored in a vacuum-desiccator over calcium chloride in a dark place. Standards of definite "percentage" can be prepared on the assumption that 1 mg. of the powder is equivalent to 7 mg. of the fresh gland. To prepare the standard the powder is extracted with 0·25 per cent. acetic acid by grinding in a mortar. The suspension is then boiled and filtered.

Only a brief summary of the methods for standardisation has been given. Two other methods have been suggested: firstly, the observations of the pressor effect, and secondly, Spaeth's method consisting of observing the contraction produced in the melanophores of Fundulus heteroclitus, using potassium chloride as a standard. The former methods have been criticised by many workers (Hamilton and Rowe (95), Heidelberg, Pittenger and Vanderkleed (96)), in that the pressor effect depends upon the algebraic sum of the actions of the pressor and depressor substances. The second method has not been used to any extent.

For a full account the reader is referred to the work of Burn and Dale (*loc. cit.*), 1922, already mentioned.

The Chemical Nature of the Active Principle

The chemical and physical properties of preparations made by various processes outlined in an earlier section are rather indefinite. In the majority of cases, the original publications dismiss the matter by stating that the material is soluble in water, and possesses all the activities of a decoction of the posterior lobe. In some cases, *e.g.* Fühner's paper, an indication of the properties has been given, but even if all the information derived from these articles be put together, it is found to be very superficial. Thus they can be summarised as follows: A

dialysable, crystalline substance, soluble in water, alcohol and butyl alcohol. It also gives Pauly's reaction for the iminazol ring.

Many attempts have been made to isolate a pure substance. In addition to those mentioned under processes of manufacture, a few additional references may be made. Thus Aldrich (97) in 1908 prepared a crystalline picrate and a sulphate ; Houssay (98) in 1911 described an active crystalline substance ; Engeland and Kutscher (99) in 1911–12 isolated guanine, choline and other substances from pituitary extracts. All attempts to obtain a pure active substance have failed. Abel and Pincoffs (100) in 1917 examined Fühner's hypophysine (Farbwerke Hoechst Co. product) very carefully. The four "pure" substances were found to contain relatively large quantities of contaminating albumoses, peptones and other protein-like materials. Their properties are as follows :

Substance 1.—A colourless crystalline sulphate, soluble in water, but soluble with difficulty in alcohol, acetone and ethyl acetate. The picrate is sparingly soluble in water. It is lævo-rotatory ($(\alpha)_D = -54{\cdot}02°$) and gives the Pauly and the biuret reactions. Physiologically, it acts slightly on the uterus, but typically upon the blood pressure.

Substance 2.—Physical properties similar to above, except that it is soluble only in water with an acid reaction. It is lævo-rotatory ($(\alpha)_D = -27{\cdot}17°$) and gives the Pauly and biuret reactions. The picrate is easily soluble in water. With alkalis a volatile amine is liberated. The substance has a marked action upon the uterus.

Substance 3.—A crystalline sulphate present in minute amounts. It is easily soluble in water and acid methyl alcohol. It is lævo-rotatory ($(\alpha)_D = -39{\cdot}25°$). The Pauly and the biuret reactions are positive, and the picrate is soluble in water. The physiological reactions are similar to No. 2, except that the oxytocic action is more powerful.

Substance 4.—This is obtained by evaporating to dry-

ness the mother liquor from which the above substances have been fractionally crystallised. It consists of a brittle glassy mass, with solubilities similar to the above. The specific rotation is $(\alpha)_D$ 21·26 : and the Pauly, but not the biuret reaction is positive. The picrate is sparingly soluble in water. The oxytocic properties are present, but there is no action on blood pressure or respiration.

Protein-like contaminants were isolated from all fractions. Abel and Pincoffs claimed that the albumoses, etc., present were quite sufficient to account for the positive Pauly and biuret reactions, and also for the lævo-rotation. A series of commercial extracts was examined, with similar findings.

The Pauly Reaction.—The significance of a positive Pauly reaction has been carefully considered by Aldrich (101). Pauly first described the reaction in 1904 (102), when he showed that an alkaline (sodium carbonate) solution of histidine gave a typical diazo reaction with diazotised sulphanilic acid. The deep cherry-red colour still persists in dilutions of 1 in 100,000. A large number of protein hydrolysis products were tested with this reaction, and it was found that only tyrosine and histidine gave a positive result. The original technique is as follows :

1. *Preparation of Diazo Benzene-sulphonic Acid.* Two grams of finely powdered sulphanilic acid are ground up with 3 c.c. of water and 2 c.c. of pure hydrochloric acid until a thick paste is formed. One gram of potassium nitrite dissolved in 1-2 c.c. of water is added in small quantities, cooling between each addition. The addition should be made in less than one minute. The sulphanilic acid passes rapidly into solution, and a thick white crystalline precipitate of the diazotised acid separates out. This is filtered off and washed with cold water. Unchanged sulphanilic acid is stated not to influence the reaction.

2. *The Reaction with Histidine.*—The presence of tyrosine must first be excluded by performing Millon's test. An excess of sodium carbonate is added, followed

by 3-5 c.c. of a freshly prepared alkaline carbonate solution and a few centigrams of the diazo compound. Almost immediately a dark cherry-red colour appears, which can be turned to an orange tint by the addition of acid. Inouye modified the reaction in order to detect histidine in the presence of tyrosine. He showed that whereas histidine in combination with other bodies, such as benzoic acid, will give a positive Pauly reaction, tyrosine will not. He therefore shakes the solution with a few drops of benzoyl chloride until the odour of this body disappears. The solution is then made alkaline, and the Pauly reaction is proceeded with in the usual manner. If positive, histidine is present. Histidine in combination with the protein molecule will not give a positive reaction unless hydrolysis is performed.

Aldrich (*loc. cit.*), applying this knowledge, found that histidine-like bodies were always present in desiccated posterior-lobe tissue and in extracts. He emphasised strongly the fallacies in arguing that histidine itself was present. He states : " Pauly's reaction is not a specific reaction for histidine, unless other compounds, such as tyrosine, oxyphenylethylamine, histamine, adrenaline, etc., are removed ", and " the compounds giving Pauly's reaction are probably not histidine, since Weidel's reaction, as modified by Fischer, or Knoop's reaction with bromine were both negative ".

The work of Führer (*loc. cit.*) suggested that there might be more than one active principle in the hypophysis, and that the pressor, depressor and oxytocic actions might be due to three separate bodies. Herring (103), 1914, supported this contention, and suggested the presence of three separate principles.

The state of knowledge up to 1914 can be summarised by stating that the active principle or principles were known to be dialysable, of a protein or protein-like nature, and to give the Pauly and biuret reactions.

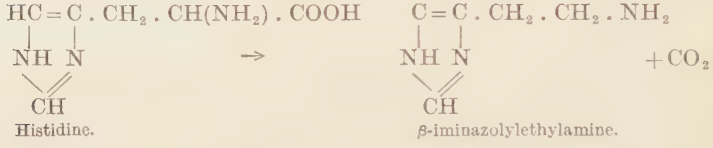
The clearest way to describe the ensuing progress will be to bridge over the work of the intervening years to 1919, when Abel and Kubota (35) published an account of

experiments with the object of proving that β-iminazolylethylamine is the oxytocic and depressor substance secreted by the gland. Immediately following the appearance of this paper, a whole series of other publications arose, in which this theory was severely criticised, both by original work and by a recapitulation of older observations.

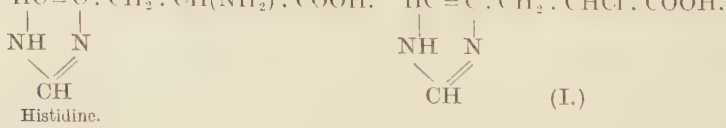
Since histamine has played such an important part in the whole question of pituitary extracts, it will not be out of place to give a short account of its chemistry and of its pharmacological properties.

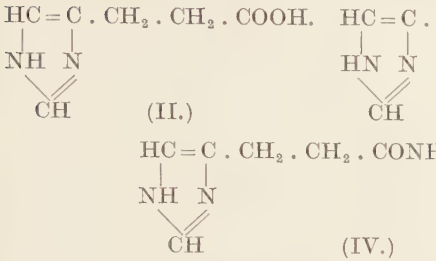
β-iminazolylethylamine. 4-(5-)β-aminoethyl-glyoxaline. Histamine

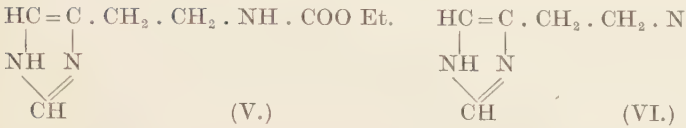
Chemistry.—β-iminazolylethylamine is the amine derived from histidine by the loss of the carboxyl group:



It was first prepared in 1907 by Windaus and Vogt (104), who used as the starting-point of their synthesis iminazolyl-propionic acid (II.). This substance can be prepared either from histidine itself or synthetically. In the preparation from histidine, the amino-acid is first converted into α-chloro-β-iminazolyl-propionic acid (I.) by the action of sodium nitrite and fuming hydrochloric acid; this substance is reduced to β-iminazolyl-propionic acid (II.), which is then converted to the ester (III.), the hydrazide (IV.) and, finally, to the azide and urethane of the base.

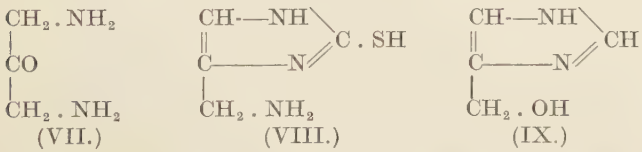






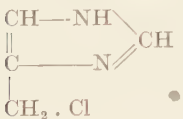
This last step is accomplished by Curtius' method, by treatment with amyl nitrite and hydrochloric acid in alcoholic solution. Hydrolysis of the urethane (V.) by concentrated hydrochloric acid yields the hydrochloride of the base (VI.).

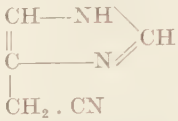
β-iminazolylethylamine has also been synthesised by Pyman (105). The starting-point of Pyman's synthesis is diamino acetone (VII.), a substance obtainable in good yield from the reduction of di-isonitrosoacetone (Kalischer, *Ber.*, 1895, 28, 1519). From this, by heating with an equimolecular proportion of potassium thiocyanate, Pyman obtained 2-thiol 4- (or 5-) aminomethylglyoxaline (VIII.) in 64 per cent. yield. The latter, on oxidation with nitric acid, is also acted on by the nitrous acid formed during the reaction, so that 4- (or 5-) hydroxymethylglyoxaline (IX.) results, nitrogen and sulphuric acid being also formed.

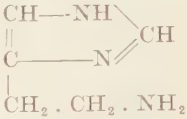


The hydroxyl group in (IX.) is readily replaced by a chlorine atom by the action of phosphorus pentachloride ; and potassium cyanide converts the resulting chlormethylglyoxaline (X.) into the cyano-compound (XI.). Reduc-

tion of the latter with sodium and alcohol yields β-iminazolylethylamine (XII.).


(X.)


(XI.)


(XII.)

Histidine may be directly decarboxylated by bacterial action, as was shown by Ackermann (106). The yield is satisfactory, and this method is the most practical one for the preparation of the base.

For this purpose Ackermann (*loc. cit.*) dissolved histidine hydrochloride (49 g.) in water (4 litres) and added 10 g. of Witte peptone, 20 g. of glucose, a few drops of solutions of magnesium sulphate and sodium phosphate, and an excess of calcium carbonate to prevent the solution from becoming acid. The solution was inoculated with a little finely minced ox-pancreas, which had been incubated for twenty-four hours previously in a weakly alkaline medium, and the whole was kept for fifty-two days at 35° C. After this period the β-iminazolylethylamine can be precipitated as the picrate, on the addition of saturated picric acid solution. This dipicrate $C_5H_9N_3(C_6H_3O_7N_3)_2$ (m.p. 238–242° C. corr.) is very sparingly soluble in cold water, and can be recrystallised from hot water.

The yield of the dipicrate is approximately 40 per cent. of the theoretical. From the mother-liquor of the picrate 0·57 g. of iminazolyl-propionic acid can be isolated as the chloroplatinate. The picrate is the most convenient compound for the isolation and identification of β-iminazolylethylamine, which can be recovered from the dipicrate by splitting the latter with hot dilute hydrochloric acid and extracting the picric acid with ether. The hydrochloride of the base then remains in the aqueous solution. It is extremely soluble in water and sparingly soluble in alcohol. The dihydrochloride has the formula $C_5H_9N_3 \cdot 2HCl$ and crystallises in prisms (m.p. 240° C.).

The base, like histidine, gives Pauly's reaction with diazotised sulphanilic acid, and Knoop's reaction with bromine water on boiling. It may be distinguished from histidine, when the latter is present in polypeptides or proteins, by the absence of the biuret reaction. The behaviour on benzoylation is characteristic, for the glyoxaline ring is broken, leading to the formation of the compound :

$$\begin{array}{l} \diagup\!\!\diagup CH \cdot NH \cdot CO \cdot C_6H_5 \\ C\!\!-\!\!NH \cdot CO \cdot C_6H_5 \\ \diagdown CH_2 \cdot CH_2 \cdot NH \cdot CO \cdot C_6H_5 \end{array}$$

a tribenzoyl-butentriamine. Histidine under similar treatment yields a monobenzoyl derivative.

The *physiological actions of histamine* have been fully investigated by Barger and Dale (107) and Dale and Laidlaw (108). They showed that dilutions of 1 : 25,000,000 were quite sufficient to produce a definite contraction of the uterus. The intravenous injection of 1 mg. of histamine into an animal will produce marked contractions of the uterus *in situ*. Atropine does not change these effects, but late in pregnancy the sensitiveness to β-iminazolylethylamine is markedly diminished. Since the contractions seem to be produced without any relation to nerve-supply and are inco-ordinate, the drug cannot have any expulsive effect upon the uterine contractions. Barger and Dale (*loc. cit.*) also demonstrated that the depressor effect of Popielski's vaso-dilatin (a substance prepared from intestines) was due to histamine, which causes a marked fall in blood pressure, together with dilatation of the peripheral vessels. Lim and Schlapp (109) have shown that histamine stimulates the flow of gastric juice.

Histamine then possesses two of the physiological actions of pituitary extracts, namely, the oxytocic and depressor actions, and, in addition, gives the Pauly reaction. It is not surprising, therefore, that speculation as to the chemical nature of the active principles of the hypophysis should have been centred upon histamine. Such an idea had evidently occurred to Guggenheim (110),

1913, since he describes certain differences between the chemical reactions of the two bodies. Thus he proved that whereas histamine is stable to the action of dilute alkalis, the oxytocic properties of pituitary extracts is rapidly destroyed by similar treatment.

As already stated, however, Abel and Kubota (35) in 1919 made the statement that they had isolated histamine from the posterior lobe, and that this substance was the oxytocic and depressor principle. Both chemical and pharmacological evidence against this theory was advanced. Perhaps the most convincing experiments were made by Hanke and Koessler (111), who were unable to isolate histamine from perfectly fresh glands, but if glands were allowed to stand for some time, they had no difficulty in detecting its presence. It could always be found in dried glands, and they suggested that the amounts recorded by Abel and Kubota were due to putrefactive changes. Hanke and Koessler state that the methods of slaughterers and manufacturers are such as to favour these changes.

Comparisons between Histamine and Pituitrin.—Dudley (*loc. cit.*) obtained abundant evidence of the chemical differences between histamine and pituitary substances. The following summary is taken from his paper:

Pituitary Uterine Stimulant.	*Histamine.*
Readily extracted from acid (HCl) solutions by butyl alcohol under reduced pressure.	Only very slowly extracted under similar conditions.
Rapidly destroyed by N. NaOH at room temperature.	Unaffected under similar conditions.
Rapidly destroyed by trypsin.	Unaffected by trypsin.
Insoluble in boiling chloroform.	Soluble in boiling chloroform.

The only point of similarity observed between these two bodies is that both are readily extracted from alkaline solutions by butyl alcohol.

Dudley also noted that the oxytocic principle was more easily extracted from hydrochloric acid solutions

than the pressor principle. In addition to the chemical evidence a number of pharmacological investigations have demonstrated differences between the action of histamine and pituitrin. Thus Cow (112) showed that whereas histamine relaxes the uterus of the mouse, pituitrin contracts it. Other differences in action were also found. Abel and Macht (113) replied to the criticisms advanced by Cow. The difference in action was explained by stating that Cow employed too weak a solution of pituitrin and too strong a preparation of histamine. They showed that strong solutions of both bodies paralyse plain muscle, whilst weak solutions have a stimulating action. On repeating Cow's experiments, using solutions of corresponding strength, they were unable to detect any difference between the action of the two bodies.

Dale and Dudley (114) made still further comparisons between the two bodies. They proved that the oxytocic action of pituitary extracts could be destroyed by boiling with 0·5 per cent. hydrochloric acid (confirming the work of Abel and Nagayama). By this treatment the activity could be reduced by one-fifth in thirty minutes. Even after this hydrolysis one two-hundredth of the activity remains, and the oxytocic power is greater than that of a corresponding dose of histamine with equal depressant powers. Again, the oxytocic principle of the pituitary extract is slowly destroyed by erepsin, but neither it nor the pressor compound is affected by pepsin. Dale and Dudley therefore conclude that even if histamine is present in the glands, the amount is so small as to be unrecognisable and unimportant. Differences in the uterine response to these two bodies were also pointed out by Burn and Dale (M.R.C. Special Report No. 69).

Judging from their recent publications Abel and his co-workers seem to have modified their views to some extent in the light of these criticisms. Thus in the paper by Abel and Macht (*loc. cit.*) the concluding paragraph of the summary says: "The hypothesis that histamine may be present in the posterior lobe of the pituitary gland in two forms, (*a*) as a histamine compound, (*b*) as free

histamine in equilibrium with the compound is now being put to the test in this laboratory". Nagayama (115) investigated a series of protein hydrolysis products obtained from various sources (thyroid, etc.) and compared their toxicity and action upon the uterus. He found marked amounts of histamine like substances present in all active material, as judged by the quantity of chloroform-soluble material present. In the same year, 1920, Abel and Nagayama (116) conducted a very careful investigation into the occurrence of histamine in the pituitary tissues. They state that perfectly fresh glands contain histamine, even when untreated by such drastic methods as long boiling in acid solutions, and that, moreover, all commercial extracts for clinical purposes examined contained appreciable amounts of histamine.

The depressor action, they state, is due to two substances: one a histamine-like body, giving the Pauly, but not the biuret reaction. It is soluble in alcohol, but not in chloroform: the second substance is histamine itself. "The histamine-like substance, and histamine as well . . . are apparently not specific constituents of the infundibulum". This is borne out by the work of Nagayama already referred to. The plain muscle stimulating oxytocic substances can be extracted with tetra-nitro-aniline, with which they form a condensation product. It is many times more powerful in its action upon the uterus than histamine. Abel and Nagayama regard the pressor and oxytocic actions as being due to one substance. Still later, in 1922, Abel and Rouiller (117) described a continuation of the researches on the lines of the previous communication. They propose the following method for preparing a relatively pure, highly active oxytocic and pressor compound.

The entire pituitary glands were placed in cold storage overnight immediately after removal from the skull. The following morning the posterior lobes were separated and ground to a very fine paste. To 100 g. of this paste, 100 c.c. of a 0·35 per cent. solution of hydrochloric acid and 4 g. of powdered mercuric chloride were added, and

the mass was well shaken for a few minutes. A bottle was then filled with the mixture so as to leave only a very small air space between the material and a tightly fitting cork. The next step is as follows : 10 g. of finely powdered mercuric chloride are added for each 100 g. of original posterior lobe paste. The cork is again replaced, and the bottle put in a shaking machine and shaken gently for two hours. It is then placed on ice. The next day, a heavy precipitate which contains the pressor and oxytocic principles has been deposited. This heavy precipitate, together with all the insoluble portions of the posterior lobe, such as connective tissue and proteins, and also such other substances as are precipitable by saturation with mercuric chloride under the conditions above mentioned, are collected at the pump and pressed to a very hard cake. The dry cake is then disintegrated by triturating in a mortar with a saturated solution of mercuric chloride.

The mass is again pressed to dryness, and the dry cake is once more ground up in a mortar with a 1 per cent. solution of mercuric chloride. The mixture is then pressed to dryness on a Büchner funnel, and is set aside in a stoppered bottle. Twenty g. of this cake are ground up in distilled water, and after neutralisation to litmus with 2 per cent. sodium hydroxide (total volume of water and sodium hydroxide = 100 c.c.) it is treated with 2·5 g. of powdered sodium chloride to produce coagulation. The mercury compound is decomposed with hydrogen sulphide, and after filtration with suction the solution is aerated to remove hydrogen sulphide, and is treated with 2 per cent. sodium hydroxide until the solution is still barely but perceptibly acid to litmus. (About 6 c.c. of the alkali solution will be required.) The volume of the solution is now about 98 c.c. As the total volume of water and reagents used in working up the cake was 106 c.c., 8 c.c. of solution is lost in the various manipulations. For purposes of calculation it is assumed that each cubic centimetre of the solution is equivalent to 41·0/106 = 0·387 g. of fresh glands. Throughout all the

above operations the solutions are kept cooled with ice water.

One cubic centimetre of the solution thus obtained is diluted 10,000 times, and 12 drops of the diluted solution are equivalent in their action on the guinea pig's uterus, suspended in 40 c.c. Locke's solution, to 10 drops of a 1 : 100,000 solution of histamine phosphate. One c.c. of the original solution is therefore equivalent to 0·083 g. of histamine phosphate, and 1 g. of the fresh glands is equal in oxytocic power to 0·083 0·387 = 0·215 g. histamine phosphate.

Ten c.c. of the original solution, when evaporated in a platinum crucible and heated to constant weight at about 110° C., left 0·3434 g. residue, which was ashed with concentrated sulphuric acid, the ash thus obtained weighing 0·3742 g. Assuming that the only inorganic matter in the original solution was sodium chloride, this 0·3742 g. ash (sodium sulphate) would correspond to 0·3080 g. sodium chloride, and hence the amount of organic matter in the 10 c.c. of solution would be 0·3434-0·3080 = 0·0354 g. or 0·00354 g. per c.c., and therefore equivalent in oxytocic power to 0·083 0·00354 = 23·5 times its weight of histamine phosphate.

It would appear, therefore, that Abel now considers that the oxytocic substance is a more powerful compound than histamine. Jaeger (118), in a paper on substitutes for ergot, gives a list of substances capable of stimulating the uterus, and compares the activity of such substance as tyramine with that of pituitrin and histamine.

Dudley (119), 1923, describes the preparation of a crystalline picrate from the active principle of the gland. Its activity, however, was about equal to that of histamine, and, on careful examination, it was found to consist of an inert compound (possibly a creatinine picrate) contaminated with traces of the active substance. From his observations he concludes that there are at least three different active compounds in the posterior lobe.

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CHAPTER III

THE INTERNAL SECRETION OF THE THYROID (IODOTHYRIN, IODOTHYREOGLOBULIN, THYROXIN)

Historical

THE early history surrounding the thyroid gland is mainly concerned with its anatomy. Theories as to its probable function were wild and imaginative, and were entirely without any experimental basis. A full review of the early literature is given by Swale Vincent (1).

General interest was first aroused by Sir William Gull's (2) description, in 1874, of thyroid atrophy associated with definite clinical symptoms and signs. Three years later Ord (3) confirmed these observations, and suggested the name myxœdema. Following the earlier extirpation experiments of Raynard (4), V. Rapp (5) and others, experimental physiologists began to work at the functions of the gland.

Chemistry

The chemistry of the gland received practically no attention until 1895, when Kocher (6), impressed by the beneficial effects of iodine in cachexia thyreopriva, suggested that this element might be present in the gland.

Prior to this Macadam (7), 1854, had attempted to find iodine in the body tissues, but had failed. Tschirsch (8), working under Kocher, also failed to demonstrate its presence in the thyroid. Almost at the same time Baumann (9) published an account of his researches, in which he claimed to have shown that thyroids from

human subjects and animals contained appreciable amounts of iodine. These investigations were rapidly extended, both by Baumann and others, with the result that iodine was invariably found in the thyroid gland.

Many complete analyses of the gland were performed (Morgenstern (10), Zunz (11), Fenger (12), Schulz (13), Labat (14)). The following elements were found: C, H, O, N, S, P, Na, K, Ca, Si, As, F, Cl, Br, I, and possibly Mg. Proteins, fats and carbohydrates, urea and purines are present in variable quantities. The thyroid is also stated to be rich in lipase (Yushchenko (15)).

By far the most important question is the occurrence of halogen compounds. Since tissues, other than the thyroid, contain at a maximum 0·001 per cent. of iodine, whilst the gland itself contains fully ten times this quantity, it is only natural that the majority of workers should have devoted their attention to the compounds of this element.

One of the first difficulties lay in the estimation of minute amounts of organically bound iodine. Of all the methods, it is probable that only three (Hunter (16), Bourcet (17) and Kendall (18)) are sufficiently accurate. A satisfactory method will be described in detail later. Iodine compounds are known to occur in certain forms of deep sea life, such as algæ, corals and the like. The table on following page, reproduced and modified from Cameron's article in *Endocrinology and Metabolism*, shows the iodine content of the thyroid in various animals.

A long series of investigations has been performed to show the various factors influencing the iodine content of the gland. Zunz (*loc. cit.*), working with fresh thyroids obtained from victims of the war, found that the iodine content was much higher than that of glands from people dying of some disease. His results are as follows: extremes, 0·044 to 0·426 per cent. of dry, or 0·011 to 0·121 of wet gland: total iodine content of whole glands gave extreme figures of 0·00315 to 0·0449 g.: mean, 0·01553 g. Age and sex appear to affect the percentage, since Monery (19) stated that the maximum was to be found between the ages of forty and sixty years, whilst Cameron (20)

Animal.	Sex.	Number.	Average Weight. Kg.	Average Weight of Thyroid.		Average Percentage of Water in Thyroid.	Average Iodine Content of Thyroid. Mg.	Iodine in Dried Thyroid (not Fat Free). Per Cent.	Mg. Dried Thyroid Tissue per Kg. Animal.	Mg. of Thyroid Iodine per Kg. Animal.
				Fresh. g.	Dry. g.					
Dogfish (squalus)	M.	75	2·3	0·091	0·0144	84	0·030	0·210	6	0·013
	F.	37	5·1	0·183	0·0297	84	0·056	0·189	6	0·011
Pigeons	Mixed	24	0·27	0·040	0·0058	76	0·027	0·477	21	0·099
Crows	,,	9	0·32	..	0·0088	..	0·065	0·751	27	0·202
Guinea-pigs	,,	15	0·44	0·058	0·0113	80	0·013	0·113	26	0·028
Dogs	,,	11	16·9	3·73	1·011	73	0·982	0·098	60	..
Hogs	,,	200	..	8·8	2·90	67	7·83	0·24	..	..
Sheep (American)	,,	200	..	8·9	1·96	78	1·18	0·06	..	..
,, (English), 1912	,,	3280	..	2·83	0·83	71	2·59	0·343	..	..
,, ,, 1913	,,	6964	..	2·60	0·62	76	2·46	0·407	..	..
Cattle	Bulls	470	636	30·0	9·0	70	19·4	0·215	15	0·042
,,	Steers	1068	545	23·0	8·1	65	19·7	0·244	17	0·060
,,	Pregnant cows	1123	454	24·4	7·5	69	18·5	0·247	17	0·060
,,	Non-pregnant Cows and heifers	1021	420	22·6	7·0	69	17·0	0·243	17	0·059
Man	M.	15	70	..	6·22	..	5·40	0·086	89	0·077
,,	F.	26	60	..	4·85	..	4·31	0·089	81	0·072
,,	Male (normal)	..	..	..	8·5-59·2	75	15·53	(0·2)	..	..

suggests that female glands contain more than male. Seasonal variations are also stated to occur. Thus Seidell and Fenger (21) found that the content was much greater in the months between June and November than in other parts of the year. Variations in the temperature are advanced as causes for these changes, since in colder weather the animals perform more exercise, and consequently the active principle would be drained away from the gland: in summer, a storage would take place. One of the most important factors is the administration of iodine in the food, and all workers are agreed that this forms one of the most rapid means of raising the iodine content of the gland. First worked out by Baumann and Goldmann (22), many other investigators have confirmed the observations on this point (Roos (23), Simpson and Hunter (24)). Fish diets are said to increase the value very markedly. Marine (25) showed that iodine administered either orally or subcutaneously was rapidly removed from the circulation by the thyroid gland. Since iodine feeding causes a marked increase in the colloid, it would be reasonable to suppose that the iodine compound is located mainly in this substance (Cameron and Carmichael (26), Fordyce (27)). With regard to the actual nature of the halogen compound, little was known until recently. As early as 1896 Drechsel (28) stated that iodine found in marine organisms was in the form of an iodoamino acid, whilst in 1916 Okuda and Eto (29) confirmed this observation by demonstrating that it was all in the soluble, non-protein fraction.

It was suggested that the compound itself might be di-iodotyrosine, but Oswald (30) proved that only a small percentage of the total iodine was represented in this fraction. Paraiodophenylalanine was suggested by Wheeler and Clapp (31) as being the iodine compound, but no observer has succeeded in demonstrating the presence of either of these bodies in the thyroid. That the greater part of the iodine in the gland is in organic combination has been proved by Blum and Grützner (32). From 1897 a series of iodine-containing bodies was pre-

pared from the gland, and it will, perhaps, be as well to describe them in order.

I. Iodothyrin or Thyro-iodine

Preparation.—This compound was first described by Baumann (*loc. cit.*), who thought at first that he had isolated the active principle. The method of preparation is as follows: after freeing the tissue from fat it is boiled up with four times its weight of 10 per cent. sulphuric acid for from four to eight hours. By this time only a slight reddish precipitate will be left. This is filtered off, and is boiled with 90 per cent. alcohol, which in turn is filtered off and evaporated to dryness. The resulting product is mixed with ten times its weight of lactose, and the paste is extracted with petroleum ether. The undissolved portion is dissolved in sodium hydroxide solution, and the insoluble matter is filtered off. Iodothyrin can be precipitated in a fairly pure condition from this solution by the addition of sulphuric acid. Further purification may be effected by repeated solution in alkali and precipitation with acid.

This method has been modified by von Fürth and Schwarz (33). They reflux thyroid tissue for ten hours with 10 per cent. sulphuric acid; the undissolved precipitate is separated and is boiled for twenty hours with 85 per cent. alcohol, and the undissolved material is filtered off. A large excess of ether is added, and a syrupy precipitate separates. This is collected, and is digested with a solution of sodium hydroxide. The insoluble material is filtered off, and the iodothyrin is precipitated by the addition of sulphuric acid. After settling, the supernatant liquid is decanted, and the precipitate is washed with water, filtered off, and dried *in vacuo* over sulphuric acid at room temperature. The dried product is extracted with petroleum ether to remove fat and is desiccated again.

Chemical Properties.—The substance, as prepared by either of the above methods, is a brownish, amorphous

solid, insoluble in water. It is soluble in concentrated mineral acids, in acetic acid, and in dilute alkalis. It is said to be insoluble in cold absolute alcohol, but is soluble in hot neutral alcohol. The compound dissolves freely in chloroform and acid alcohol, but is only slightly soluble in ether and ethyl acetate. If ether is added to a chloroform solution, the iodothyrin can be precipitated as a flocculent mass. The ordinary alkaloidal reagents precipitate it from any of its solutions. That the iodine is strongly bound is shown by the fact that sulphuric acid and sodium nitrite fail to split off any iodine. Fixed alkalis and sodium amalgam appear to remove the iodine if allowed to act over a very long period. Boiling baryta water, however, produces no change. Heating for many hours in an autoclave at a pressure of six atmospheres is sufficient to liberate the iodine. The residue will then give Millon's and Ehrlich's aldehyde reactions for the first time. The elementary composition varies. Roos (*loc. cit.*) gives the following figures: S, 1·40; N, 8·91-10·41; C, 57·04-61·41; H, 7·28-7·43; Ash, 0·4-0·47; Cl, 0·4-0·05 per cent.

Iodothyrin contains about 10 per cent. of iodine. This figure is very variable. The compound forms about 4 per cent. of the total weight of the dried gland. Blum (34), 1898, regards the substance as an inconstant fission product of the gland proteins. Perhaps one of the most interesting points concerning iodothyrin is that Nürnberg (35), 1907, working with the compound, suggested that the iodine was present in the form of an iodised tryptophane.

Physiological Properties.—Baumann regarded this substance as the active principle of the gland, and stated that it possessed all the therapeutic properties of thyroid tissue. He seemed to regard its activity as being greater than that of dried gland. Some workers confirmed his views, whilst others failed to do so. A full account of the clinical trials up to 1911 is given in *Abderhalden's Biochemisches Handlexikon*. It would appear, however, that the evidence as to its potency compared with that of dried gland is rather confusing.

II. Iodothyreoglobulin

Preparation.—This substance was isolated by Oswald (36). The glands are defatted and dried, or the extraction may be made with wet tissue. A watery extract is made and is filtered. The clear filtrate is half saturated with ammonium sulphate, when a precipitate appears. This is allowed to settle and is collected and dried.

Chemical Properties.—Iodothyreoglobulin possesses all the properties of a globulin, except that it contains iodine. This varies markedly in amount. Thus preparations from pigs' glands contain 0·46 per cent., from ox 0·86 per cent., and from human thyroids 0·34 per cent. The substance is fairly rich in sulphur (Nürnberg), the proportion of which varies from 1·83 to 2·0 per cent. The compound forms between 14 and 60 per cent. of the weight of the dried gland (Wiener (37), 1909). In very young animals the substance may be iodine free. The compound can absorb iodine from the blood stream, and the iodine content can be increased by iodine administration. By employing repeated extraction with 0·9 per cent. sodium chloride solutions, Oswald (*loc. cit.*) proved that he could remove all the iodine from the gland. After removal of the iodothyreoglobulin by half saturation with ammonium sulphate, he was able to precipitate a nucleoprotein by full saturation. This compound contains no iodine. The elementary composition of iodothyreoglobulin is stated to be as follows : C, 52·2 ; H, 6·7-6·96 ; N, 16·51-16·67 ; I, 1·57-1·75 ; S, 1·77-1·45 (see above) ; O, 20·85. The molecular weight is stated to be in the region of 8000.

It is sparingly soluble in water, but easily soluble in dilute salt solutions, dilute acetic acid and dilute alkalis. If a solution of the substance be boiled with 5 per cent. hydrochloric acid, large quantities of carbohydrate are split off. Digestion with liquor pepticus liberated a body very similar to iodothyrin, containing about 5 per cent. of iodine, whilst a tryptic digest dissolved the substance and removed the iodine. Since large quantities of tyrosine

are present in the solution, it would appear that the iodine is not in combination with this body. Oswald came to the conclusion that the iodine was united to the indole ring.

Physiological Properties.—The compound is stated to be active, but no reference to quantitative experiments could be found.

Many other substances have been isolated. These include Fränkel's thyreoantitoxin, Notkin's (39) thyroprotein and various preparations of Drechsel (*loc. cit.*). None of these, however, has received any extensive physiological trials, and they are consequently without much interest.

Asher (40) has described experiments with a substance called "thyreo-glandol", which is prepared by perfusing fresh thyroids with normal saline. Although the preparation is stated to contain no protein and very little iodine, its activity is said to be very marked. It would appear, however, that the methods of testing used by Asher were very unreliable. There are various German preparations on the market called "thyroidin", which probably consist of Baumann's iodothyrin.

III. Thyroxin

No further advances were made until Kendall (41) in 1915 published his first paper dealing with the isolation of an active, crystalline body from the thyroid gland. This substance was ultimately named thyroxin.

Before describing Kendall's processes in detail, it will perhaps be as well to indicate the steps leading up to the elaborate methods used later. In the first place, it must be realised that the iodine in the thyroid tissue cannot be separated by dialysis. Kendall (*loc. cit.*) set to work to determine by what chemical means the iodine could be rendered dialysable. A number of drastic methods suggest themselves, but all proved useless for this particular process. After many trials it was decided to employ mild alkaline hydrolysis by means of alcoholic

sodium hydroxide. This type of method was originally suggested by Vaughan (42), but was considerably modified by Kendall. By this means 75 per cent. of the iodine becomes dialysable.

Another interesting point is that half of the iodine compounds produced by thorough alkaline hydrolysis are soluble in acid alcohol, whilst the insoluble material is to a large extent non-dialysable.

If a solution of the acid-soluble material be made alkaline, the colour deepens to a brownish black, and the solution then gives all the typical tests for a colloidal solution (Tyndall phenomenon, etc.). In the actual process the acid-soluble material is used. It is dried and rendered free from fat by extraction with ether. The actual separation is carried on from this stage, using certain properties of the barium salts to effect a separation.

Kendall's Method of Preparation.—The following detailed account is a summary of the U.S. Patents 1,392,767 and 1,392,768. The method described in the second is a distinct improvement upon the first, since fresh thyroids can be used, and the necessity for alcoholic sodium hydroxide is done away with.

Kendall emphasises the importance of alkali-resisting containers, and recommends that vessels for the hydrolysis, etc., should be made of nickel. The actual description can best be conveyed in a series of steps.

1. A mixture of thyroid glands, water and sodium hydroxide is made in the following proportions :

Fresh thyroids . . .	1 part by weight
Water	3 parts by weight
Sodium hydroxide . . .	$\frac{1}{6}$ part by weight

(or any other metallic hydroxide to make about a 1·25 N solution). The sodium hydroxide must be first dissolved in the water, and the thyroids added next. The container, pipes, etc. should be of nickel. The contents are brought to the boil, and are agitated by blasting steam through a nickel-plated U-tube in the liquid. The boiling is continued for twenty-four hours.

2. After cooling, 5 kg. of sodium chloride are added for every 10 kg. of glands. Solution is rendered complete by stirring, and the resulting liquid is syphoned off into a suitable vessel. On standing, fat and impurities can be skimmed off the surface. The clear liquid is syphoned off from under any superficial solid layer which may have formed after twenty-four hours. If the fluid is not absolutely clear, any flocculent material must be filtered off through cloth filters, or a centrifuge machine may be used. The final solution is red and slightly syrupy.

3. This solution is acidified with 50 per cent. sulphuric acid (by volume). After thorough stirring for some time, a flocculent precipitate separates and sinks to the bottom. After twenty-four hours the supernatant liquid may be separated, and if clear it may be discarded: if not, any remaining precipitate is collected and added to the first. The precipitate is mixed with infusorial earth, and after filtration on a Büchner funnel it is washed with a little distilled water.

4. The precipitate on the Büchner funnel is now dissolved in dilute sodium hydroxide. The proportion is judged as follows: after the alkaline hydrolysis already described, the solutions from 20 kg. of glands are combined for acid precipitation. The acid precipitate, after washing, is dissolved in 1 litre of water and just enough NaOH is added to leave a slight excess present. The solution is then filtered to free it from infusorial earth and certain sulphur compounds which separate at this point.

5. This alkaline solution is acidified with hydrochloric acid, the temperature not being allowed to rise above 25° C. After stirring, the precipitate is allowed to settle, and is filtered off either with or without infusorial earth as before. It is dried in the air at room temperature.

6. This dry precipitate containing excess of hydrochloric acid is added to 1000 c.c. of 85-95 per cent. alcohol (presumably for 20 kg. of thyroid — not mentioned in patent). The solution is assisted by the addition of

hydrochloric acid in small amounts (10 to 15 c.c.) until a distinctly acid reaction results. Large excess should be avoided. Insoluble material is filtered off and kept separately.

7. The clear alcoholic solution is neutralised to blue litmus paper with 30 per cent. sodium hydroxide solution. After stirring, the resultant tarry, black precipitate (No. I.) is allowed to settle for twelve hours. The liquid is filtered off and is acidified with hydrochloric acid until it just turns blue litmus red.

8. Barium hydroxide is stirred into this alcoholic solution until it becomes alkaline. It is then refluxed for a non-specified time. This causes the appearance of a precipitate (No. II.), which is removed, after cooling, by filtration.

9. 100 c.c. of a 5 per cent. aqueous suspension of barium hydroxide are added to the alcoholic solution from precipitate No. II., and the alcohol is distilled off. The aqueous residue is filtered and the precipitate is collected (No. III.). Thyroxin is distributed between precipitates I., II., III. and filtrate from III. Precipitate No. I. contains such a small quantity of the active material that it is not worth while pursuing. Precipitates II. and III. and filtrate from III. are worked up.

Kendall classifies the problem of separation as follows :

Group I. Compounds insoluble in water in the presence of barium hydroxide.

Group II. Compounds soluble in water in the presence of barium hydroxide.

Group III. Precipitates obtained by passing carbon dioxide through the solutions after removal of the barium.

Group IV. Precipitates obtained by acidifying solutions with hydrochloric or sulphuric acids.

Group V. Precipitates obtained from the (supernatant) fluid of Group III.

10. Precipitates II. and III. are each suspended in 400 c.c. of water, boiled for half an hour at 100° C. and

filtered. A separation into Groups I. and II. of the above scheme has thus been effected.

11. The insoluble precipitates may be combined, and dissolved in 400 c.c. of water containing 20 c.c. of 30 per cent. sodium hydroxide solution. The contents of the beaker are brought to the boil. By this means the water insoluble barium salt of thyroxin is extracted presumably as a sodium salt, and, after filtration, it is found that all the iodine is present in the filtrate. Kendall summarises the treatment of water insoluble barium compounds of Group I. as follows :

Insoluble barium compound.
+
H_2O and NaOH
↓
Heat—Boil on hot plate.
↓
Filter —→ { Filtrate is the same as the soluble barium compounds of Group II.
↓
Residue + HCl
Filter
↙ ↘
Residue saved. Filtrate discarded.

12. *Group II. or Soluble Barium Compounds.*—This includes the filtrates from steps 10 and 11. Sodium sulphate is added to precipitate the barium as sulphate. No excess of sodium sulphate should be present. The barium sulphate is now filtered off on a Büchner funnel, and the temperature of the filtrate is adjusted to 25° C.

13. Carbon dioxide is passed through the solution until a precipitate appears. If, after an hour, there is no precipitate, the alkalinity is reduced by the addition of hydrochloric acid. If, again, no precipitate results, the solution is rendered distinctly acid with hydrochloric acid, and the resulting precipitate is separated off and worked up as in Group IV. The carbon dioxide precipitate is filtered off and belongs to Group III. The scheme is as follows :

Soluble barium compounds $+Na_2SO_4$

$BaSO_4$ separates. Filtered off.

Filtrate $+CO_2 \rightarrow$ (1) ppt. $\rightarrow$ Group III.

(2) no ppt., add HCl ppt. $\rightarrow$ Group IV.
Filtrate discarded.

14. Group III. consists of precipitates produced by passing carbon dioxide through the solutions resulting from steps 11 and 12. The purity of these precipitates can be judged from their colour—the darker the more impure. Precipitates are combined according to their order of purity, so as to make as few batches as possible. They are dissolved separately in 200 c.c. of water containing 15 c.c. of 30 per cent. sodium hydroxide. The solutions are brought to the boil.

15. Sodium chloride is added in such a quantity as to bring the percentage in the solution up to 20. After cooling, a crystalline or tarry precipitate separates out. This is the sodium salt of the iodine compound (thyroxin), which has been rendered insoluble by increasing the percentage of sodium chloride. If all has gone well, it will be crystalline, but certain impurities render the precipitate tarry. The precipitate is filtered off, and after cooling by the addition of ice the filtrate is acidified with hydrochloric acid, and the resulting precipitate is reserved for material under Group IV.

Group III. precipitates, obtained by passing CO_2 through alkaline solutions of Groups I. and II.

Dissolved in 30 per cent. NaOH, then NaCl to make concentration up to 20 per cent.

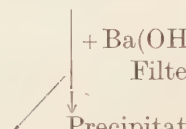
Ppt. (*a*) of Na salt of iodine compound (thyroxin).
Filtrate cooled with ice, acidified with HCl.

Ppt. (*b*) $\rightarrow$ Group IV.
Filtrate discarded.

16. Group IV., precipitates resulting from acidifying solutions after precipitation with carbon dioxide, and after treatment as in Group III. The precipitate is dissolved in a small volume of dilute sodium hydroxide (200 c.c.). After transferring to a nickel crucible and acidifying with hydrochloric acid, 10 g. of barium hydroxide are added. The crucible is heated in an electric oven at 100° C. for eighteen hours, loss from evaporation being reduced to a minimum. A precipitate forms, which is filtered off, and is treated as for Group I. (insoluble barium compounds).

The filtrate is treated as for Group II. (soluble barium compounds). The process is now recapitulated as from step 10.

HCl ppt. dissolved in NaOH and reprecipitated with HCl.



17. *Group V. Treatment of Precipitates from Group III.*—It will be remembered that under Group III. two precipitates were obtained: (*a*) the sodium salt of thyroxin and (*b*) a precipitate which is carried to Group IV. As the process goes on the quantity of (*a*) increases at the expense of (*b*), and it can be seen that eventually the material will consist of the sodium salt precipitated in Group III. Group V. deals with the purification of such precipitates. Crystals from Group III. are dissolved in 200 c.c. of dilute sodium hydroxide. The solution is heated to 100° C. on a water bath for one hour. A flocculent, dark, iodine-free precipitate comes down and is filtered off. The filtrate is then treated as in Group IV. After prolonged treatment NaCl precipitates from Group III. will begin to take on a highly crystalline form. They may be purified as follows: the crystals are dissolved in 85-95 per cent. ethyl alcohol containing a trace of sodium hydroxide. After acidification with acetic acid

the solution is boiled, when a fine white crystalline precipitate separates out. This is filtered off, redissolved in 3 per cent. sodium hydroxide, and sodium chloride is added to bring the concentration to 20 per cent.

The resulting precipitate is collected, and the whole process is repeated until a fine white crystalline product is obtained. The white precipitate from the acetic acid solution is thyroxin, the active principle containing 65 per cent. of iodine.

Na salt of thyroxin dissolved in 90 per cent. alcohol with trace of NaOH.

↓ Acetic acid—heat.

White precipitate of thyroxin.

↘ Ppt. thyroxin.

↓

Filtrate—remove alcohol by distillation—proceed as in Group IV.

Attention is called, in the patent, to the solubility of the dimetallic salts of thyroxin ; the monometallic salts on the other hand are sparingly soluble, especially in carbonate solutions. A method of precipitation may be effected on these lines by taking the monometallic salt and dissolving it in alcohol containing a little sodium hydroxide over the amount necessary for solution. Carbon dioxide is passed through. A precipitate, mainly of impurities, separates out, and after filtering this off, the alcohol is removed by distillation and boiling. If allowed to stand for five to six days a precipitate of the monometallic salt settles. There are, however, several difficulties, since the separation depends on the impurities, and the time factor is variable.

Kendall attributed the success of the method to a careful study of the factors tending to bring about destruction of the compound. These precautions are as follows :

1. The temperature must be carefully watched during precipitation with acids.

2. Nickel containers and apparatus must be used for alkaline hydrolysis, otherwise iodine will be split off the compound.
3. The carbon dioxide treatment must be carried out in every detail.
4. The temperature during the passage of carbon dioxide must be maintained as stated.

Owing to the complexity of the method the diagram on the following page has been added by the authors in the hope that some sort of impression of continuity may be obtained by the reader.

Having obtained this iodine compound Kendall investigated its properties and constitution.

Physical and Chemical Properties of Thyroxin

Thyroxin occurs in colourless, odourless crystals. It is insoluble in watery solutions of all acids, including carbonic acid. It dissolves readily in sodium, potassium or ammonium hydroxides, but only sparingly in sodium or potassium carbonates. It is sparingly soluble in water. Ultimate analysis yielded the following figures : C, 22·37 ; H, 1·65 ; O, 8·73 ; N, 2·23 ; I, 65·02 per cent. This suggested the formula $C_{11}H_{10}O_3NI_3$, which gives a percentage composition corresponding very closely with the above.

The sulphate of thyroxin precipitated by means of sulphuric acid was found to have an iodine content of 60 per cent. By calculation the molecular weight of thyroxin was therefore proved to be 585, thus supporting the above formula.

For various reasons about to be discussed, the following formula was adopted.

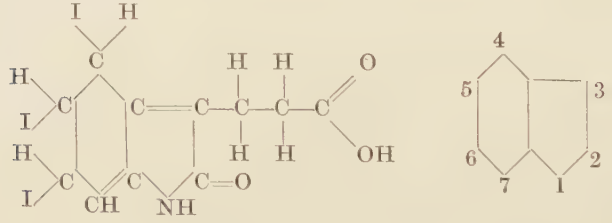
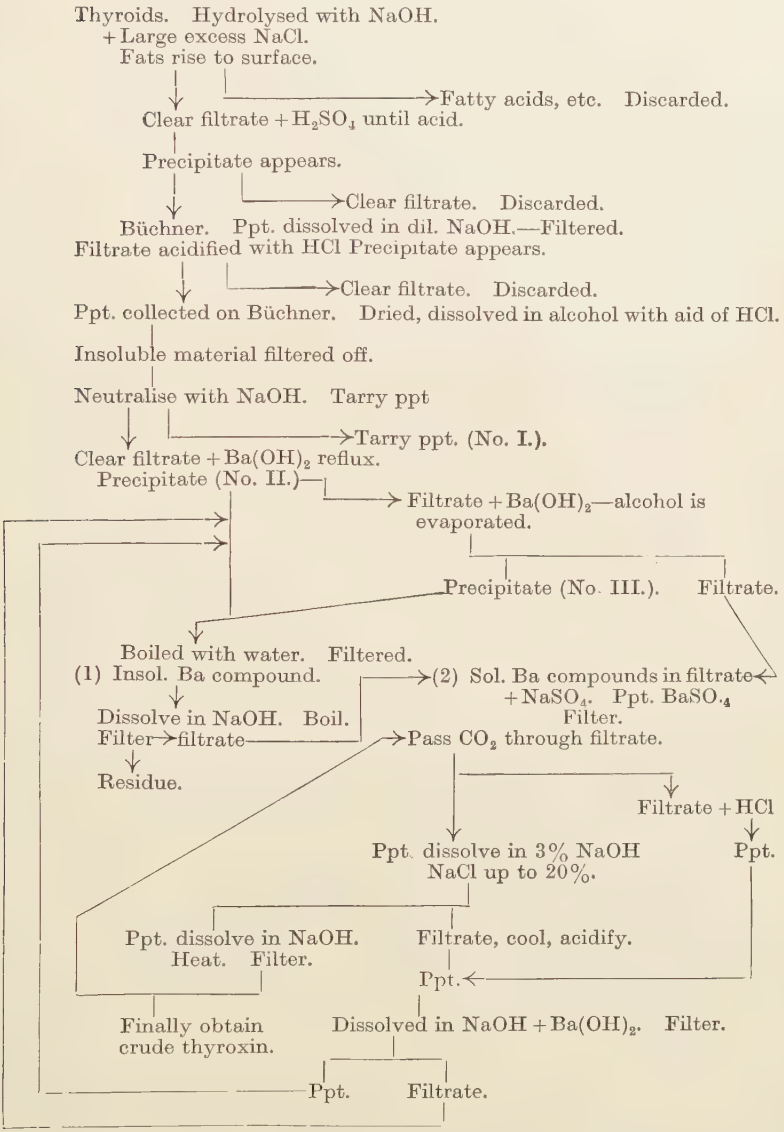


DIAGRAM SHOWING KENDALL'S METHOD FOR THE PREPARATION OF THYROXIN

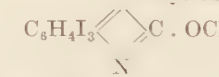


Employing the system of numbering shown in the diagram above, the compound is therefore 4-5-6 tri-

hydro 4-5 6 tri iodo 2 keto-β indole propionic acid. The reasons for assigning this formula are as follows :

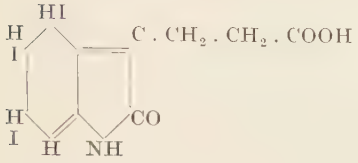
1. The presence of the *indole nucleus* had been known for some considerable time. "Thus its solubility in alkali metal hydroxides, but not in carbonates, pointed to its phenolic nature" (Kendall).

2. *Carboxyl and Hydroxyl Groups.*—The presence of these groups is considered proved by the salt-forming properties of thyroxin. Metallic salts of crystalline character can be prepared. Analysis of the silver salt shows that two atoms of silver have been added, one to the carboxyl group, and the other to the hydroxyl group. Further evidence of the existence of a hydroxyl and a carboxyl group is provided by the formation and properties of a dimethyl ester. This can be formed by adding methyl iodide to the silver salt. One of the two methyl groups is easily removed by hydrolysis with alkali, whilst the other is firmly bound. The formula of the dimethyl compound is therefore considered to be as follows :

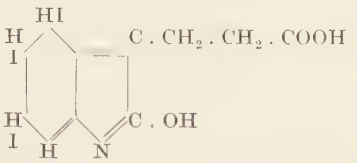


3. *Imino Group.*—The presence of this group can be proved by several reactions. If acetic anhydride be added to an alkaline alcoholic solution of the substance an acetyl derivative can be precipitated by the addition of sulphuric acid. The acetyl derivative is soluble in most organic solvents, whilst thyroxin itself is practically insoluble. Another compound involving the imino group is the ureide, formed by the interaction of a salt of thyroxin and cyanic acid. The iodine percentage in this compound corresponds closely to the formula :

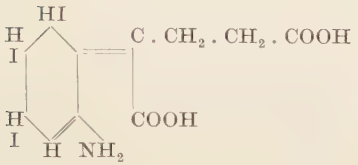
From a series of observations Kendall suggested that the substance could occur in any of the following varieties :



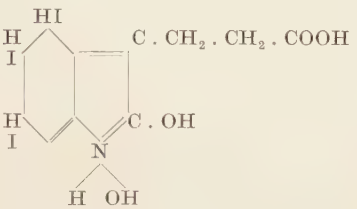
1. Keto form.
m.p. 250° C.



2. Enol form.
m.p. 204° C.



3. Open-ring form.
m.p. 225° C.



4. Amino hydrate.
m.p. 216° C.

The existence of the keto form is considered as being established, owing to the formation of the ureide with cyanic acid, and of acetyl and formyl ureides with acetic anhydride and the chloride of formic acid respectively. Furthermore, the compound does not react with hydrazine, which is " strong evidence of the carbon of the carbonyl group being the α-carbon to the imino group ". If the keto form be obtained in alkaline solution, a hydrogen atom migrates from the imino group to the adjacent carbonyl group, thus forming a hydroxyl compound. This theory is supported by a study of the monomethyl derivative, and also by the fact that in the disodium salt the second sodium atom is joined to a hydroxyl group.

If the acetyl derivative of the imino group be made, then the hydroxyl group cannot be formed by migration of a hydrogen atom on to the carbonyl group. If this compound be dissolved in water, the pyrrol ring opens with the fission of the CO—NH linkage leading to the formation of a secondary carboxyl group from the carbonyl radicle. Kendall suggests that in the body similar changes go on, and that the imino and carbonyl groups

give place to carboxyl and amino radicles, with fission of the pyrrol ring.

With regard to the position of the three iodine atoms, Kendall says, "As no special difference was demonstrable between the reactivity of three atoms of iodine, it seemed most probable that they were all attached to the benzene ring, and, as three extra hydrogen atoms would be required, if the iodine was added to, and not substituted for, hydrogen on the ring, they also were placed on the ring."

In the keto form thyroxin is insoluble in organic solvents other than strongly acidic or alkaline media. It is also amphoteric, and in addition to forming the salts described, *i.e.* sulphate and hydrochloride, it gives rise to crystalline mono-salts (Na, K, NH_4) and di-salts (as Na, K, NH_4, Ba, Ca, Mg, Ni, Zn, Cu). These are so unstable that they are decomposed with boiling water.

The enol form exists in alkaline solutions. Kendall prepares it by hydrolysis, in the cold, of a pyridine solution of the ammonium salt. On the addition of water the compound separates in needles (m.p. 204° C.).

The open-ring form is made by adding sulphuric acid to an alkaline solution of thyroxin. A precipitate forms, and is filtered off and boiled with distilled water. The open-ring form separates in "bundle-blades" (m.p. 225° C.). It is soluble in alcohol, but on standing the keto form separates out.

The fourth or amino-hydrate variety can be obtained as fine crystals (m.p. 216° C.) separating from a heated mixture of thyroxin in sodium hydroxide solution, on the addition of 10 per cent. ammonium chloride. If it is suspended in water containing a trace of formic acid, the open-ring form is produced.

As Kendall points out, the final proof of the constitution lies in the synthesis. This, he says, has been accomplished by Osterberg, but no details have, as yet, been published.

Tests for Thyroxin.—Any of the reactions quoted above might be used as tests. A rough test consists of adding

nitrous acid to an alcohol solution, or to an aqueous suspension. A trace of hydrochloric acid must be present. A yellow colour develops, which is turned pink on the addition of ammonia. The compound is fairly easily reduced. Sunlight splits off the iodine if the compound is present in weakly alkaline solution. Hypoiodous acid (HIO) is liberated and, finally, free iodine. The solution also turns black.

The compound has no action on polarised light; this may be due either to the absence of an asymmetric carbon atom or to the fact that the process of preparation gives a racemic product. (A full account of this work is given in Kendall's original papers (43-49).) In conclusion, a few words must be said concerning the yield. In 1919 Kendall stated that 33 g. of thyroxin had been prepared from 6650 lb. of thyroid. The poor yield, taken with the complexity of the process of manufacture, renders the production of thyroxin on a commercial scale impracticable.

The physiological action will be dealt with at a later stage. Suffice it to say here that all workers are agreed that thyroxin possesses all the actions of whole thyroid glands.

Before proceeding to a discussion of the action of thyroid substances, it will be as well to summarise what has gone before. It has been shown that one of the most striking characteristics of thyroid tissue is its high iodine content. Kocher and others had called attention to the beneficial effects of iodine in cases of thyroid deficiency, consequently iodine became regarded as forming one of the elements of the active principle. It has also been noted that the activity varies roughly with the iodine content.

A series of bodies has been isolated, the most important of which are Baumann's iodothyrin, Oswald's iodothyreoglobulin and, most important of all, Kendall's thyroxin. The latter contains 65 per cent. of iodine, but even then only accounts for a quarter of the iodine in the gland. The physiological action of thyroid administration will

now be very briefly reviewed, after which methods of testing the potency of preparations will be dealt with.

Physiological Action of Thyroid Preparations

The most important action of thyroid tissue, fed by the mouth, is its ability to restore cretins and myxoedematous patients to normal. The basal metabolic rate increases, and, with suitable dosage, the return to normal is complete. (For a full account see text-books of medicine.) Phosphorus and nitrogen excretion is increased, and the body weight diminishes if the dose is pushed. The substance acts by a general stimulation of metabolism. In common with iodine compounds in general, it accelerates the metamorphosis of tadpoles but retards the growth of frogs. The growth-rate of rats and rabbits is retarded, which is a specific effect. The liver is rendered glycogen free, and the resistance to certain poisons is increased (*e.g.* acetonitrile). Very large doses may kill the animal. Previous administration of thyroid is said to increase the pressor effect of adrenaline. This has been used as the basis for standardisation (Asher).

With regard to the preparations described, various authorities have reported differently upon the activity of iodothyrin. Thus, Baumann and Roos stated that its action was more rapid than that of dried gland. Other work supporting this claim is that of Baumann and Goldmann, Hilderbrant (50), Irsai (51), Magnus-Levy (52). Those who failed to confirm the above observations were Notkin (39), Wormser (53), Hennig (54), Asher and Flack (55). The general conclusion is that the reaction is variable. Iodothyreoglobulin is supported clinically by Magnus-Levy, Courvoisier (56), Lanz (57) and Oswald.

Iodothyrin is said to be less potent : the same applies to other iodine-containing proteins derived from ocean sources, such as gorgonin and spongin : iodotyrosine and iodocasein are without action.

The action of thyroxin has been worked out very carefully. Kendall states that there is considerable delay in

the action. Successive daily administrations of thyroxin bring about death, whilst the injection of single doses of enormous size fails to cause any demonstrable changes. Kendall concludes that the molecule *per se* is non-toxic, but that the substance acts as a general metabolic catalyst. Large doses are rapidly excreted, over 56 per cent. being accounted for in the urine and bile.

With regard to its general action, the catalytic theory is supported by all the known facts. The action is additive, and takes place within the tissues. Its action lies in stimulating the metabolic processes rather than in causing the appearance of new ones, and therefore its absence results in a slowing down of metabolism rather than cessation and death. Thyroxin, apparently, was added to the body economy in order to " permit a greater range of flexibility of energy output " (Kendall). The catalytic action is said to depend probably upon the CO-NH grouping. Thyroxin, it is maintained, possesses all the actions of thyroid. Passage through the alimentary canal does not affect its activity.

There can be no doubt that thyroxin is the most active principle yet obtained, but it would appear that it is only one of several substances present in the gland which are responsible for the action.

Methods of Standardisation

The difficulty facing the investigator is that there is no specific chemical test for the active principle. Again, there is no simple physiological reaction which could be made the basis of a method of assay.

Asher's Biological Tests.—Asher (58) investigated the activity of thyreoglandol by means of a biological method. Using the Laewen-Trendelenburg heart strip preparation he made the thyroid intensification of the action of adrenaline the basis of his test. His work has been criticised by Romeis (59). Since the reaction of tadpoles has been shown to be produced also by iodides, no method of assay can be based upon such a test.

Hunt's Acetonitrile Test.—Reid Hunt (60) evolved a reaction known as the acetonitrile test. He demonstrated that the resistance of white mice to acetonitrile (CH_3CN) was markedly increased by feeding with thyroid extract. The method is open to many fallacies, and, moreover, the protecting power of the substance appears to vary directly with its iodine content.

As already pointed out in an earlier section, the activity of the material varies directly in proportion to the iodine content, and it would appear that the best method of assay would be in the estimation of the iodine content. The only fallacy in such a method would be the addition of iodides to the preparation. The estimation of iodine is the only method in general use at the present time. The American Pharmacopœia demands that all preparations shall contain not less than 0·2 per cent. of iodine, 0·003 per cent. latitude above and below this figure being permitted. The Dutch authorities demand the presence of 0·4 per cent. of iodine, whilst in England no limit is fixed.

The estimation of iodine in small quantities of tissue was formerly a very difficult matter, but owing to the work of Kendall "iodine estimation is one of the most accurate methods of analysis of small amounts of any element in biological material that has been devised" (Cameron).

Since this method has found universal favour it alone will be described in detail.

Kendall's Method for Determining Iodine in the Thyroid (61)

The first step is the destruction of organic matter and the retention of the iodine as sodium iodide. This is accomplished by fusion with sodium hydroxide in a nickel crucible. In order to maintain a proper degree of temperature and to prevent loss of iodine by volatilisation, the crucible is heated by placing it inside a larger crucible, the bottom of which is covered with a layer of sand 0·5 cm. in thickness.

The supporting cylinder is 9·4 cm. in diameter and 30 cm. high ; the cross bars which support the large crucible are 7·5 cm. from the top. The larger crucible is of iron, and is 7·8 cm. in diameter. The smaller one is of pure nickel and is 5·9 cm. in diameter. The burner is preferably a 15·6 cm. (No. 3) Meker burner.

For the determination of iodine in thyroid preparations it is best to use not more than 0·5 g. of material. Whether this is in the form of a dry powder, a solution or a moist precipitate on a filter paper, the same procedure is carried out. The material is placed in a 5·9 cm. nickel crucible and moistened with a few drops of 30 per cent. sodium hydroxide ; 5 to 10 g. of stick sodium hydroxide which has been broken into small pieces are added, and the crucible is placed on a hot plate until the excess of water is evaporated and the contents have a thick, syrupy consistency. If but little organic matter is present, there is a tendency for spattering of fine drops during the evaporation of the excess water. If some organic substance is dissolved in the solution this spattering is prevented. A small amount of lactose is suitable and sufficient for this purpose.

For the fusion of the organic matter with the sodium hydroxide it is necessary to heat the bottom of the large crucible to a red heat. If the crucible is heated too much the contents of the small crucible will creep up the sides and will volatilise, with loss of iodine. If the large crucible is heated insufficiently the destruction of organic matter will not be complete. However, there is a wide range of temperature between the two limits, and after a little experience no difficulty is encountered.

When the sodium hydroxide is first heated in the presence of water considerable foaming is produced. This, however, does not extend more than half-way up the sides of the crucible. As the heating continues the foaming becomes less, and after from five to ten minutes the melt settles to the bottom. Bubbles continue to be given off for some time (five minutes or so), depending on the nature of the organic matter.

When the melt has settled to the bottom and only a few bubbles of gas are being liberated, the small crucible is removed with crucible tongs and partially cooled by agitating the contents with a rotary motion. This will also remelt and carry to the bottom any particles of the fusion which have solidified on the cooler sides of the crucible.

Five to ten mg. of potassium nitrate are now added. This oxidises the remaining organic matter and causes a liberation of bubbles. If only a few bubbles appear, a second addition of from 5 to 10 mg. of potassium nitrate will not cause a further liberation of bubbles, and the oxidation of the organic matter is complete. If the second addition of the nitrate causes a further oxidation, repeated additions of from 5 to 10 mg. of the nitrate are made until no more bubbles of gas are produced by the addition of the nitrate. The melt is now poured into the shallow cover of the 5·9 cm. crucible and allowed to cool.

The entire time required for the fusion is from ten to fifteen minutes. It is most convenient to use two crucible-supporting cylinders and to carry on two fusions at the same time.

When the melt and the crucible are cool they are placed in a beaker of from 600 to 800 c.c. capacity, and 125 to 150 c.c. of water are added. The beaker is then placed on a hot plate. After the melt is dissolved it is transferred to a 500 c.c. Erlenmeyer flask. It should be a colourless, clear solution, with a volume of about 200 c.c. To this solution 5 c.c. of 20 per cent. sodium bisulphite and *two drops only* of a saturated aqueous solution of methyl orange are added. The solution is cooled by immersing the flask in cold running water. When it is cool 85 per cent. phosphoric acid is added by allowing the acid to run directly into the flask from a pipette or syphon having a small delivery tube. The flask is vigorously and constantly shaken with a rotary motion to expel the carbon dioxide. As the indicator begins to turn pink the neutralisation is finished slowly, and the addition is stopped at the first definite change of the indicator to

pink. Five c.c. of 20 per cent. phosphoric acid and a small piece of hard coal, about 0·5 cm. in diameter, are added. The volume is adjusted to about 250 c.c. and the water is boiled on a hot plate for at least ten minutes, and longer if necessary, until the volume of the solution is about 200 c.c. After the flask has been cooled in water bromine is added, and the solution shaken until the bromine imparts a distinctly yellow colour. This is essential, as the addition of too little bromine will prevent the subsequent determination of iodine. The flask is again placed on a hot plate and the solution is boiled, the time when all visible bromine is expelled being noted. This will require about three minutes of actual boiling. The solution is boiled for just five minutes after it is colourless. It is then removed from the hot plate, a few crystals of salicylic acid are added, and the flask is immersed in cold water. The volume of the solution after boiling should not be less than 175 c.c., as the high concentration of the salts makes the end-point less sharply defined. (*Note.*—A considerable error in the determination of iodine may result if any distilled water is added after the bromine has been boiled out of the solution.) Five c.c. of reduced 20 per cent. phosphoric acid and about 1 g. of pure potassium iodide crystals are added.

The liberated iodine is now titrated with 0·005 N sodium thiosulphate. The titration is finished with the aid of the blue starch-iodine colour. For this a 0·5 per cent. solution of soluble starch is recommended.

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CHAPTER IV

THE INTERNAL SECRETION OF THE OVARIES

Historical

That the ovary is concerned with sexual characteristics has been known for a long time, but it is only within the last twenty-five years that any attempt at ovarian therapy has been made. It is possible that Knauer (1) in 1900 was the first to produce definite experimental evidence that the ovary was concerned with the phenomena of œstrus, and that the results of spaying an animal could be in part overcome by an ovarian graft. He was not, of course, the first to demonstrate the atrophy of the uterus and tubes following removal of the ovaries, a fact universally known to farmers and breeders for many years previously. Knauer, however, was the first to show that these symptoms could be overcome by transplanting an ovary from another animal. Simultaneously, Theodore Landau (2) began to give dried ovaries by the mouth for relieving symptoms of the climacteric, and for those following double oöphorectomy in younger women. These results were repeated by Mainzer (3) in 1903. He described a series of cases in which the symptoms of artificial menopause produced by removal of the ovaries had been greatly benefited by the administration of ovarian tissues. In one case the results were so astounding that the patient was given, without her knowledge, scraped meat as a control. All the symptoms returned, and were relieved when the original treatment was restored. Mainzer prepared his substance by drying minced pig and cow ovaries for twelve hours at a temperature of between 60° C. and 70° C. The dry material was

then made into tablets, which represented the whole of the ovarian tissues. As will be seen later, this distinction is of the greatest importance, since many of the later "improvements" merely consisted in removing what is now believed to be the active principle.

Knauer's experiments were fully confirmed by later workers such as Marshall and Jolly (4), with the result that great interest was shown in ovarian therapy. Fränkel (5), 1903, stated that he had obtained good results by the administration of corpus luteum. All these investigators gave their preparations by the mouth, although Federoff (6) reported in 1899 the results of injecting ovarian extracts both subcutaneously and by the intravenous route. The only observation he made was that the pulse rate was lowered. One of the most peculiar points about the early literature is that none of the observers referred to, with the exception of Knauer, attempted any animal work. It seems an astounding thing that these substances should have been administered to human patients without preliminary trials upon spayed animals, where the observations could have been easily and thoroughly controlled. It was not until Adler (7) in 1912 published an account of his work upon the effect of injecting animals with ovarian preparations that there was any attempt to observe with accuracy the results of gland therapy. This observer stated that he was able, by repeated injections of watery extracts of whole ovaries, to produce menstrual periods and typical signs of œstrus in animals. He checked his observations by histological investigations of the uterine mucosa, and fully confirmed his earlier statements. This work must undoubtedly be regarded as a turning-point in the history of the subject, since it is the only scientifically controlled series of investigations performed up to this date.

Methods of Preparation

The year 1912 saw a series of complicated chemical investigations published. Thus Iscovesco (8) published

an account of the preparation of an active substance by extracting ovaries with alcohol. He obtained a lipoid, soluble in alcohol and ether, from both the ovary and the corpus luteum. His work gave the stimulus for pure chemical investigation, and very shortly afterwards a whole series of similar researches was reported. Fellner (9) published a paper almost immediately after the appearance of Iscovesco's, in which he independently described a method for extracting ovaries with volatile solvents. He minced the ovaries, extracted with alcohol, and subsequently evaporated off the solvent. The residue was suspended in saline, and was injected into ovariectomised rabbits. Typical symptoms of œstrus such as hyperplasia of the vagina and uterus were produced. It can be seen that Fellner's work was a very great contribution, for not only did he show that the hormone was thermostable, and soluble in alcohol, ether and acetone, but he also devised a sound method for testing the products. A lapse of two years saw another series of important chemical papers. Seitz, Wintz and Fingerhut (10) gave a detailed account of the preparation of lipoidal material from the corpus luteum. Their method, taken from the German patent No. 320,857, 19 7 1914, can be summarised very briefly as follows: freshly peeled corpora lutea are minced, and are then subjected to successive extraction with alcohol and acetone. These processes are followed by ether and chloroform extractions. After evaporation, the acetone, alcohol and chloroform extracts are combined. More chloroform is added, and the whole is allowed to stand. After some time, two layers form: these are separated and evaporated to dryness. Two lipoidal substances result, one soluble in chloroform, containing C, 61·2; H, 12·5; N, 4·13; S, 2·29; P, 2·83 and O, 16·85 per cent. Injection of this substance stops menstruation, whilst the other, a water soluble compound, promotes menstruation. The latter substance is a lecithalbumin. The patent makes no reference to the ether extract. A further patent, taken out in 1916 (D.R.P. 332,165, 25 1/16) states that the method can

also be applied to whole ovaries and placenta. An active substance, for promoting menstruation, can be prepared by extracting dried tissues with alcohol or chloroform. In the same year Okintschitz (11) prepared extracts of whole ovaries, liquor folliculi and corpora lutea by grinding with saline and glycerol. He named these products ovarin, proprovar and luteovar respectively. These extracts were administered to ovariectomised rabbits by subcutaneous injection. He showed that whereas the extracts of ovary and liquor folliculi were active, that from corpus luteum was quite inactive.

Herrmann and Fränkel's Method of Preparation.— Without doubt, the most careful and painstaking chemical work was done by Herrmann and Fränkel (12) in 1915. This method has been patented in Germany, England and America. The following account is taken from the English patent No. 113,311, 13/2/1918.

Finely minced ovaries are extracted several times with some volatile solvent — preferably alcohol. The best yields are obtained by extracting the previously dried tissues. The solid matter is separated from the solution by filtration and by pressing, and the alcohol is distilled off. This leaves a watery residue with the active material present as a suspension. This residue is then shaken up with ether in a separating funnel, and by this means the suspended material is removed from the water. The ether solution is then evaporated down, and acetone is added to precipitate the lipoidal fraction, which may be separated by filtration or centrifugation.

The clear solution is then evaporated to dryness, when the active principle is obtained. This substance promotes menstruation. It can be purified in the following manner : after precipitation of the lipoids with acetone, the solvent is evaporated off, and the residue is dissolved up in alcohol. Fatty acids are precipitated by the addition of an alcoholic solution of lead acetate. After removal of the excess lead (method not given), the solution is evaporated to dryness, and is then distilled in the highest possible vacuum. The hormone distils over between 190° and

210° C. The distillate, which in addition to the active material contains some cholesterol esters, is treated with a small volume of cold alcohol. The hormone dissolves readily, and a great deal of the cholesterol compounds crystallises out and can be separated by filtration. The alcohol is evaporated off and the oily residue is subjected to a second distillation in high vacuum.

Placenta is also suitable for the preparation of this substance.

Properties.—The substance is "a light, viscid oil at normal temperature" and distils from a bath at a temperature of 240° C. under a pressure of 0·06 mm. The temperature within the flask is 193° C. Preparations give the reactions for cholesterol, but active material can be obtained almost free from cholesterol.

On analysis it was found to contain only carbon, hydrogen and oxygen, and to give the following percentage composition : C, 81·33 to 81·62 ; H, 11·32 to 11·49.

Physiological Properties.—When injected subcutaneously it promotes the growth of the uterus, ovaries, tubes and vagina. Premature sexual maturity can be produced in prepubertal animals, and œstrus can be produced out of the rutting season.

The substance has been administered clinically, but we were unable to find any details of the results in the literature.

It is indeed astonishing that so complete a piece of work should have received so little attention, and, in so far as we could ascertain, no firm is making this preparation for clinical use. There can be little doubt, as will be seen later, that the substance prepared by this method is identical with that of more recent observers.

Herrmann and Fränkel, in this and other patents, suggest so many alternative methods of extraction and purification, that it would seem that they had tried every method possible for the preparation of an ovarian hormone. It would appear, however, that the above process is the one which they favour.

A series of investigations was published by Frank (13)

and his collaborators from 1915 onwards. He maintained the importance of extraction with lipoidal solvents, and prepared a series of substances similar to that of Herrmann and Fränkel. He also succeeded in obtaining physiologically active material from the corpus luteum. From this time onwards almost all reputable investigators employed volatile organic solvents, and their observations agreed in the main with those of Herrmann and Fränkel.

Hietzman's Extract of Liquor Folliculi.—In the light of recent work, that of C. M. and W. I. Hietzman (14) is of considerable interest. These observers thought that liquor folliculi and the contents of cysts seen so frequently in ovaries might contain the active principle. They patented a method which consisted of crushing the ovaries in a closed vessel, and collecting the juice which escaped. By this means the tissue can be almost desiccated. The fluid is rendered clear by filtration through fine wire gauze, pressure being applied by means of a stiff-haired brush.

From 1914 to 1923 a series of papers appeared upon the action of ovarian extracts upon various organs and conditions. Thus in 1914 Aschner (15) showed that extracts of ovaries and placenta, when injected, produced rut-like symptoms in castrated guinea-pigs. Mazzei (16) studied the effect of watery ovarian extracts upon the iris and showed that dilatation resulted. Itagaki (17) demonstrated an increased tonus of the uterus after the application of ovarian extracts. Weil (18) performed some experiments upon the effect of such injections on the respiratory exchange of animals. Abderhalden, working with Schiffman (19) and later with Gellhorn (20), isolated some bodies called optones from the ovary, and studied the effect of these upon paramœcia. During this period the whole subject of ovarian therapy gradually came into disrepute, owing to the excessive zeal of commercial firms and to the undue optimism of certain sections of the medical profession. This resulted in the good work being forgotten, and it was not until a few months ago that the subject has been re-opened on a scientific basis.

In 1917 Stockard and Papanicolaou (21) noted that the type of cells obtained from a vaginal smear of small mammals was markedly influenced by the appearance of œstrus. In prepubertal rats the vagina is represented by a solid cord of cells, and appears in the vulva merely as a slightly raised mark. The onset of puberty is shown by the opening out of this strand of cells, with the production of a definite passage. In the non-œstral rat, smears of the vagina, stained with hæmatoxylin and eosin, show the presence of epithelial cells in various stages of nuclear degeneration and cytoplasmic shrinkage, together with some polymorphonuclear cells. The onset of œstrus may be accompanied by congestion of the vulva, and the vaginal opening appears larger. The smear shows nucleated epithelial cells alone. When œstrus is at its height the vagina is almost perfectly dry. The smear shows cornified non-nucleated, red eosin-staining cells. The presence of those cells makes the diagnosis certain, even in the absence of external signs. As œstrus recedes, these changes take place in reverse order. These researches provide an accurate means of determining the onset of what corresponds to menstruation in higher animals, and consequently could be made the basis of a method of using and standardising ovarian preparations. Investigators equipped with this knowledge would be able to control their chemical researches with an accuracy approaching that of insulin standardisation by means of the effect on blood-sugar content.

The work described above would suggest that the potency of ovarian preparations could be measured by two methods. A unit could be based upon the quantity required to produce the typical signs of puberty in a rat long before the age at which that condition supervenes naturally, or, on the other hand, the amount required to induce œstrus in a castrated animal might form the basis of a method of standardisation. The former method would have certain disadvantages. Thus the date of birth of the animals would have to be accurately known, otherwise it would be difficult, even with control animals

of the same litter, to exclude the possibility of normal puberty. Also, a fresh rat would have to be used for each experiment, otherwise any positive result occurring might be explained as being due to an additive effect. If the second method were employed one animal could be used for a number of tests after the reaction had subsided. The operation of double ovariectomy in young rats is quite easy. An incision is made through the back, parallel to the quadratus lumborum, and the ovary is seized through the wound and is cut off. In large rats it is advisable to ligature the pedicle before section. The skin wound is closed with Michel's clips. Sections of the tissue should always be cut, in order to be certain that the ovaries have been removed. If this operation is performed before puberty, sexual maturity cannot be achieved by natural means. It would be expected that a "pubertal" dose would, of necessity, have to be greater than an "œstral" dose. As will be seen later, both these methods have been employed with considerable success and accuracy. The advantages of the "œstral" unit over the "puberty" unit have, however, led to its adoption.

Allen and Doisy's Method.—Commencing in 1923, Allen and Doisy (22) began to publish a series of papers dealing with these problems. The following is a description of their latest method of preparation. It will be noticed that the similarity to Herrmann's process is very striking. These investigators disagree with Herrmann on some points. Thus they support the view that cholesterol free preparations are quite potent. Herrmann always mentions that his substances gave reactions for cholesterol, but it is only fair to note that the method of purification advocated by him in the patent referred to aims at removing as much cholesterol as possible. Frank (*loc. cit.*) also maintained that active material could be obtained in a cholesterol-free condition. Allen and Doisy fail to obtain an active material from corpus luteum, which constitutes another point of difference. Thirdly, it is evident that their preparations are probably purer

than those of Herrmann, who employed 60 mg. as a dose, whereas Doisy, Ralls, Allen and Johnston (23) state that as little as 8 mg. will produce an effect. It is necessary to point out, however, that Herrmann's product was standardised on rabbits, whereas Doisy and Allen used rats for this purpose. Finally, the latter authors had the advantage of being able to use Stockard and Papanicolaou's smear method as a basis for their standardisations. It would appear, therefore, that Doisy and his co-workers have obtained a substance identical with that of Herrmann's, but in a much purer condition.

The first paper was published by Allen and Doisy (22) in 1923. The method consisted in collecting liquor folliculi from pig ovaries by means of a syringe and needle. To this fluid twice its volume of 95 per cent. alcohol is added, and, after shaking, the precipitated proteins are allowed to settle. The mixture can then be filtered. The proteins are extracted with boiling alcohol, which is subsequently filtered off. The two filtrates are combined, and the alcohol is evaporated off. An oily residue remains, which is dissolved in ether. Acetone is added, as in Herrmann's method, to precipitate lipoids, these being subsequently removed by filtration. The ether is then evaporated off, and the active principle is extracted from the residue by boiling with alcohol. After evaporating this off, the minute amount of oily residue is the active principle. This is put up for injection in purified corn oil, or is emulsified in dilute sodium carbonate solution. Injection of this material produced puberty in prepubertal rats and œstrus in castrated animals. In a later paper, by Doisy, Ralls, Allen and Johnston (23), some improvements on the process quoted above were introduced. Their present routine method is as follows: fresh liquor folliculi is mixed with two volumes of 95 per cent. alcohol. After coagulation the mixture is filtered, and the precipitate of proteins is extracted in a Soxhlet apparatus with more alcohol for six hours. The clear filtrates are combined, and the alcohol is evaporated off, either *in vacuo* or at ordinary pressure, by means of a current of

warm air. The residue, if dry, is emulsified in a few c.c. of water. A volume of 95 per cent. alcohol is now added to make the total volume equivalent to about 15 c.c. per 100 c.c. of liquor folliculi taken. The solution is now brought to the boil, and 2 volumes of acetone are added to precipitate the lipoids. After filtration, the residue is dissolved in more alcohol, and the process is repeated three or four times. By this means all phosphorus-containing substances, together with inorganic salts, are precipitated. The combined filtrates are distilled to dryness. The residue is extracted with boiling alcohol five to seven times, with subsequent cooling in a freezing mixture prior to filtration. By this technique a large amount of the inert fatty material is removed. After the alcohol has been removed by distillation the residue is taken up in ether, and is allowed to stand. A white precipitate settles out, and the clear ethereal solution of the extract is removed by centrifugation and decantation.

The final product is the residue after removal of the ether. The authors state that whole ovaries and placenta may also be successfully extracted by this procedure, although they themselves always work with liquor folliculi. All the separated fractions are inactive, and many of them are definitely toxic.

Properties.—These are almost identical with those of Herrmann's product. The authors make no mention as to whether the hormone is volatile under the conditions described by the former worker.

The substance itself is a light viscid oil, soluble in ether, chloroform, petroleum ether (b.p. 40-60° C.), alcohol (95 per cent.) and in acetone ; it is insoluble in water. It can be dissolved in oils, such as olive and corn oils, and, if cholesterol free, can be emulsified in water. An emulsion can always be obtained in weak sodium carbonate solutions. The hormone gives the reactions for cholesterol, but, by precipitation with digitonin, an active cholesterol-free substance can be obtained.

Strongly acidic or basic groups seem to be absent, as shown by the fact that it can be extracted by ether from

either dilute acids or alkalis. The purer preparations gave no biuret reaction. It is thermostable, and is fairly resistant to mild hydrolysis with dilute acids or alkalis. Tryptic digests fail to destroy its activity.

Physiological Action.—When a solution is injected subcutaneously into animals, œstrus or premature puberty results, as already described. The injections should be performed at six-hourly intervals, and within forty-eight hours a reaction occurs with potent preparations.

All the typical symptoms described previously result. The authors employ the induction of œstrus as a criterion, and the amount necessary to produce these changes in an ovariectomised rat is termed a rat unit. The weight of this varies according to the purity. As small a quantity as 0·13 mg. may effect these changes.

The yield of rat units in terms of liquor folliculi or material taken can be judged from the following table, reproduced from the paper by Doisy, Ralls, Allen and Johnston (23):

Tissue.	Rat Units per kg. of Tissue.	Weight of Rat Unit in mg.
Liquor folliculi { maximum	2180	0·13
Liquor folliculi { minimum	220	19·5
Whole ovaries	160	64
Whole ovaries. Small follicles. Non-pregnant animals	100	280
Whole ovaries. Small follicles. Pregnant animals	80	..
Ovaries minus liquor folliculi	175	84
Placenta, human	700	..

Working in conjunction with S. Wright, we have repeated Herrmann and Fränkel's and Allen and Doisy's work, and have fully confirmed their statements that an active ovarian hormone can be consistently prepared from ovaries by alcoholic extraction. We have made a detailed study of its properties, and hope to publish our findings shortly.

In conclusion, it will not, perhaps, be out of place to refer to the interesting work of Evans and Bishop (24).

These workers showed that if rats were fed on a basic diet, adequate for normal growth and health, the animals failed to reproduce. The diet consisted of the well-known casein-lard-cornstarch mixture. "The sterility produced does not interfere with the early steps of gestation. The ovulation and implantation incidence is normal, but most of the implantations are resorbed (from 80 per cent. to 100 per cent.)." Certain foods can protect against this sterility.

"Lettuce, meat, whole wheat, wheat germ, rolled oats, dried alfalfa and large quantities of milk fats are such curative substances." Evans and Bishop refer to this unknown substance as "factor X", since it does not correspond to any known accessory food factor. It would be very interesting to know whether there was any connection between the ovarian hormone and this body.

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CHAPTER V

THE INTERNAL SECRETION OF THE SUPRARENALS (ADRENALINE, ADRENIN, EPINEPHRINE)

Historical

Apart from anatomical discussions, no interest was taken in the suprarenals until Addison (1) in 1849 published an account of the clinical condition associated with destruction of these glands. This clinical entity, characterised by low blood pressure, weakness and pigmentation, at once became a recognised disease, and Addison's original observations were confirmed by many contemporary physicians, such as Wilks (2), Greenhow (3) and Trousseau (4), the last mentioned being the first to name the condition Addison's disease. In 1856 Colin (5) demonstrated that the cut surface of the suprarenals could be stained a bluish colour by means of ferric sulphate, whilst a little later Vulpian (6) extended these observations, and showed that this property was confined to the medulla of the suprarenal. He also stated that oxidising agents turned the cut surface of the gland pink, and that these microchemical staining properties extended to the suprarenal vein. From this he argued that the secretion of the gland must pass into the blood stream.

Nine years later, 1865, Henle (7) made the important discovery that the suprarenal medulla could be stained brown by means of chromic acid. He suggested the name "chromaffin tissue", a term which is still in use at the present day. From this time onwards the majority of workers directed their attention to the isolation of this chromogen, and many detailed analyses of the capsules

were made (Cloez and Vulpian (8), Marino Zuco (9)). It is, perhaps, not going too far to say that the pioneer chemical work was done by Holm (10) in 1867. This observer extracted the minced glands with weak alcohol, and, evaporating this off, he dissolved the residue in water. Basic lead acetate solution was then added to precipitate the proteins. After filtering, the excess of the reagent was precipitated by passing hydrogen sulphide through the solution. The lead sulphide was filtered off, and the chromogen was precipitated by the addition of ammonia. Holm, therefore, laid the foundations of the method for the preparation of adrenaline since he showed how to remove the proteins, and that the chromogen was precipitable by ammonia.

Eighteen years later Krukenberg (11) repeated this work, and suggested that the substance was allied in constitution to catechol. These observations formed the first suggestions as to the constitution of the substance. No new observations were made until 1894, when Oliver and Schafer (12) reawakened interest in this subject by their injection experiments. These observers stated that the active principle was confined to the medulla, was insoluble in absolute alcohol and ether, and was destroyed by alkalis; it was stable to gastric juice and was dialysable. There can be no doubt that this substance was identical with that obtained by Vulpian, Holm and Krukenberg.

The Preparation of Natural Adrenaline

Abel's Method. Apart from the earlier experiments mentioned above, the first chemical work on the isolation of the active principle was that of Abel (13), 1898–1902, who attempted to benzoylate the solution obtained by extraction of the suprarenal glands. His extracts were made with dilute acid. After concentration the extract was made alkaline, and the benzoyl derivatives prepared by the addition of benzoyl chloride (Schotten-Baumann method). The active principle was then regenerated

from the impure mixture of benzoyl derivatives by acid hydrolysis. The resulting substance was physiologically active and gave Vulpian's colour reactions, but was probably impure. In some later experiments the benzoyl derivatives were decomposed by heating with acids in an autoclave under pressure, a process which is now known to destroy the greater part of the active compound. In common with other earlier workers, Abel attempted to separate the base by the addition of ammonia to a solution which was too dilute for complete precipitation. In spite of these facts, however, he was able to obtain very active preparations of the sulphate $(C_{17}H_{15}NO_4)_2H_2SO_4$ and an impure bisulphate. The latter, for example, in a dose of 0·00013 mg., raised the blood pressure of a dog with cut vagi 14 mm. of mercury, corresponding to the effect of a tenth of the weight of the pure base.

Abel was led to the conclusion that his extract contained as an active component "epinephrin hydrate" $C_{10}H_{13}NO_3 + \frac{1}{2}H_2O$, which differed from the inactive epinephrin, which he also isolated, in that it contained a half-molecule of water. This nomenclature has led to some confusion in the literature, and later work on the pure substance has failed to confirm Abel's views. In fact, Bertrand (14) has shown that Abel's analysis fits the formula $C_9H_{13}NO_3$ just as well as the one given above.

The majority of the methods which were developed during the next few years are similar in principle, and differ only in detail. The minced glands are treated with acidulated water, and after the filtered extract is freed from protein by one or other of a variety of methods, the adrenaline is precipitated from the *concentrated* extract by the addition of ammonia. Owing to the readiness with which adrenaline is oxidised, even by atmospheric oxygen, it is desirable to protect the solution from the air as much as possible during the extraction and subsequent processes, either by a layer of oil or petrol floating on the surface or by a current of inert gas. With the same object in view the liquid used for extraction may contain dissolved sulphur-dioxide.

Takamine's Method. A process which gives satisfactory yields of the pure substance was devised and protected by English Patent No. 1467 by Takamine (15), 1901. The finely minced glands are extracted for about five hours with acidulated water at a temperature of from 50 to 80° C., with frequent stirring and the addition of water as the liquid evaporates. The temperature is then raised to 90-95° C. for one hour to coagulate the protein, a layer of fat being used to protect the surface, as is mentioned above. The liquid is pressed out and the mass is re-extracted as before. The combined extracts are concentrated *in vacuo*, and inert substances precipitated by the addition of 2-3 volumes of alcohol to the concentrated liquor. After removal of the alcohol, ammonia is added to the residual liquid until the latter is distinctly alkaline. After several hours the precipitate is collected, washed with water, and dried. This crude product may be purified by one of the processes described below.

From the figures obtained by him on analysis Takamine arrived at the formula $C_{10}H_{15}NO_3$ for the pure base. This result was not confirmed by other workers, and Aldrich's results are now accepted as being correct.

Aldrich's method of extraction (16) was published almost simultaneously with Takamine's, and is very similar, but he used lead acetate to precipitate inactive substances - adrenaline itself is not precipitated by neutral lead acetate. Aldrich obtained the figures C = 58·03, H = 7·20, N = 7·66 by the analysis of a specimen prepared by his own method, and identical figures were obtained by him from the analysis of a specimen prepared by Takamine's method and purified by him. These analyses correspond with the formula $C_9H_{13}NO_3$, which is now generally accepted.

Aldrich had previously been associated with Abel at the Johns Hopkins Medical School, and it is interesting to note that he observes that if a benzoyl residue, C_7H_5O, is subtracted from one of Abel's formulæ for epinephrine ($C_{17}H_{15}NO_4$) the formula $C_{10}H_{10}NO_3$ is obtained, which is not very far from Aldrich's own result. Jowett (17),

1904, subsequently showed by analysis that the German products suprarenin, adrenaline and epinephrin were also identical with each other and with the above substance, $C_9H_{13}NO_3$. Batelli (18), 1902, and v. Fürth (19), 1903, have devised methods which are very similar to that of Aldrich described above.

An alcoholic extraction process which gives satisfactory results is that of Abel (20), 1903. The minced glands are well mixed with about half their weight of alcohol containing about 3-4 per cent. of trichloracetic acid, and extraction is allowed to continue overnight. The filtrate is then evaporated to small volume and precipitated with ammonia. The precipitate, which is crystalline and almost white, is dissolved in dilute oxalic acid solution, and the solution precipitated with a mixture of alcohol and ether. This precipitate is redissolved in water containing a little trichloracetic acid, and the solution is purified by the addition of 800 c.c. of absolute alcohol and 150 c.c. of ether to each 50 c.c. After filtration the adrenaline is precipitated from the filtrate by the addition of ammonia.

Abderhalden and Bergell (21), 1904, extracted the glands with alcohol containing acetic acid.

In *Bertrand's method* (14) advantage is taken of the solubility of adrenaline oxalate in alcohol, but the yield is not so good as that obtained by Abel. Abel worked with bullock's glands, whereas Bertrand obtained his material from horses. The use of methylamine instead of ammonia for precipitating adrenaline is covered by the U.S. Patent 928,220 of Armour & Co., Chicago. Weidlein (22) has worked with the suprarenal gland of the whale. According to his method, to 1 kg. of the minced glands (each gland weighs from 264 to 684 g.) 550 c.c. of absolute alcohol, 50 c.c. of chloroform and 25 c.c. of acetic acid are gradually added and extraction is allowed to continue overnight. The liquid, after warming to coagulate the precipitate, and after filtration, measures 600 c.c. It is evaporated *in vacuo* to 60 c.c. and, after filtration, ammonia is added to precipitate the active

principle. The precipitate is dried with absolute alcohol and ether. The yield is about 0·15 per cent. of the pressor substance.

Purification of Crude Adrenaline

The crude extracts obtained by one or other of the above processes may be purified by Takamine's method. This consists in dissolving the material in acid and adding alcohol and ether to precipitate the colouring matter and some impurity, which are filtered off. Addition of ammonia to the filtrate yields adrenaline as a white, crystalline precipitate. It is quickly filtered, washed with water and then with alcohol, and is dried. This process of purification may be repeated two or three times if necessary.

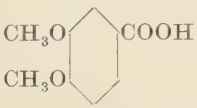
Pauly's Method.—The process of Pauly (23), 1903, depending upon the solubility of the oxalate in alcohol, a property discovered by Abel (20), 1903, is probably better. Pauly's method is as follows: the crude adrenaline (12 g.) is dissolved by careful rubbing with a glass rod in 50 c.c. of 85-90 per cent. alcohol, containing 7 g. of pure oxalic acid. The filtered solution is diluted with 100 c.c. of water and the active substance is precipitated by the addition of ammonia. The precipitate is thoroughly washed with water to free it from precipitated ammonium oxalate, but it still contains some ash. The majority of this mineral residue can be removed by solution in dilute acid (100 c.c.) and subsequent precipitation by the cautious addition of dilute ammonia. Pauly repeated this purification three times from acetic acid, twice from sulphuric and once from hydrochloric acid, and the substance then obtained gave figures on analysis agreeing closely with those required for the formula $C_9H_{13}O_3N$.

To obtain a substance absolutely free from ash the almost ash-free material (5 g.) is dissolved in 25 c.c. of 85 per cent. alcohol containing 3·5 g. of pure oxalic acid. Dry ether (15 c.c.) is added to cause a turbidity, and then

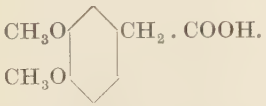
as much alcoholic-ethereal ammonia as is required to precipitate the inorganic impurities. These are filtered off, and the adrenaline is precipitated from the filtrate by the addition of aqueous ammonia. After thorough washing, the precipitate is dissolved in very dilute sulphuric acid and two volumes of a mixture of 96 per cent. alcohol and ether are added. The very slight precipitate is filtered off after about half an hour, and the adrenaline is once more precipitated from the filtrate, washed very thoroughly and is dissolved in dilute hydrochloric acid. After addition of an equal volume of alcohol, excess of free mineral acid is removed by ammonium acetate. A few drops of ammonium oxalate solution, and dilute ammonia until the reaction is weakly alkaline, are then added. If there is any turbidity, the base is again precipitated from the filtrate and reprecipitated three times from very dilute hydrochloric acid by the addition of very dilute ammonia. The yield is 3·88 g. of absolutely ash-free adrenaline from 5 g. of the almost pure substance.

The Chemical Constitution of Adrenaline

When the empirical formula $C_9H_{13}O_3N$ arrived at by Aldrich (16), 1901, had been confirmed by other workers, a study of the fission products of natural adrenaline made the solution to the problem of the chemical constitution fairly clear. Takamine had already obtained from adrenaline, by fusion with potash, catechol



(*a*) Veratric acid.



(*b*) Homoveratric acid.

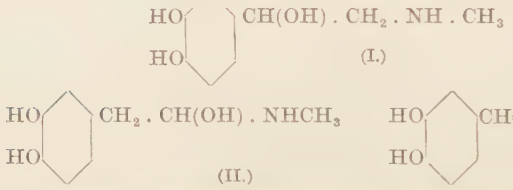
and protocatechuic acid. These observations were confirmed by Jowett and von Fürth, and the latter observer also showed that methylamine was produced by the action of concentrated acids or sodium hydroxide. Jowett, by oxidation of adrenaline with permanganate, obtained a mixture of methylamine, and oxalic and formic acids. The

same worker obtained in 1904 veratric acid (3 : 4 methoxybenzoic (*a*)) and trimethylamine by complete methylation with methyl sulphate and subsequent oxidation with permanganate. These observations show that the complex OH C and the methylimide (NH.CH_3)

OH

grouping must be present. The latter, moreover, should be found in the side chain, or methylamine would not be liberated with such readiness. The fact that adrenaline yields a tribenzoyl derivative (v. Fürth) makes it probable that a third hydroxyl group is present in the side chain. Pauly in 1903 had shown that adrenaline is optically active, and was led to believe that the secondary amino group was present, from the action of phenyl isocyanate.

From these data the probable formulæ are limited to three, viz. :



all of which contain asymmetric carbon atoms. Jowett (1904) was led to favour the first of these formulæ from the formation of veratric acid as mentioned above, and this was subsequently shown to be the correct one. Formula II. should lead to the formation of homoveratric acid (*b*) and not veratric acid (*a*) on oxidation of the methylated product. It would be difficult to explain the formation of pyrrol (observed by Abel) from a substance of formula III. This last formula was finally disposed of when Friedmann (24), 1904, 1906, supplied the proof that the hydroxyl group in the side chain is present in the form of a secondary alcohol. Friedmann showed that von Fürth's tribenzenesulphonyl-adrenaline loses its optical activity on oxidation to the corresponding keto-compound,

and this, combined with the evidence of the oxidation products, makes it clear that the constitution of adrenaline is expressed by formula I. above.

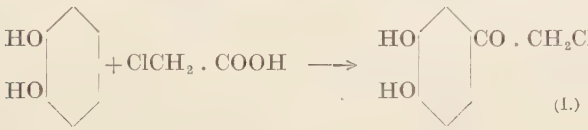
Finally this formula was confirmed by synthesis, first by Stolz and later independently by Dakin and others.

The Synthesis of Adrenaline

Among the several syntheses which have been suggested from time to time, only two seem to be suitable for the commercial preparation of synthetic adrenaline.

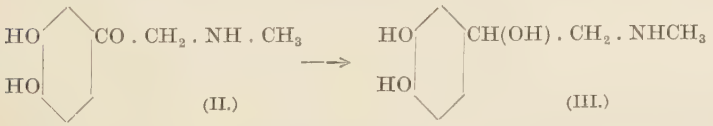
Stolz's Method.—The first of these (Stolz, 1904, D.R.P., Farb. vorm. Meister Lucius u. Brüning, Klasse 129, 152814, 155652 and 157300) begins with the synthesis of the corresponding ketone, " adrenalon ".

For this purpose catechol is condensed with monochloracetic acid in the presence of phosphorous oxychloride, leading to the formation of chloracetocatechol (I.), a substance previously prepared by Dzierzgowski :



(I.)

This substance readily combines with methylamine when twice its weight of 40 per cent. aqueous methylamine is added to a suspension of the ketone in half its weight of alcohol. The methylamino-acetocatechol (II.) separates on standing and is washed with water, alcohol and ether.



(II.) (III.)

Reduction of the latter ketone—" adrenalon " (II.) to the corresponding alcohol—racemic adrenaline (III.)—is effected by the addition of aluminium powder to the aqueous solution of the sulphate, in the presence of mercury sulphate. This reduction may also be accomplished

catalytically by means of hydrogen in the presence of finely divided platinum (25). Although the ketone (II.) shows the characteristic effect on blood pressure the action of the alcohol (III.) is very much more marked. By this synthesis dl-adrenaline is obtained as an amorphous precipitate, the resolution of which is discussed later.

By using a modification of the above synthesis with other amines in place of methylamine, a number of homologous substances can be prepared (Loewi, O., and H. H. Meyer (26), 1905; Dakin (27), 1905).

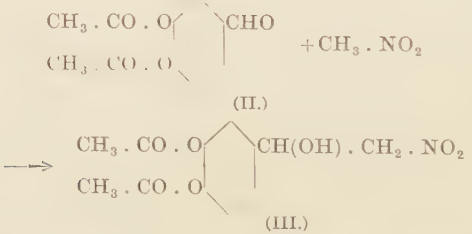
Nagai's Method.—More recently Nagai has described a process which he has protected by English Patent No. 118,298 (1918) for the production of synthetic adrenaline. According to this patent the process consists in condensing diacetylprotocatechuic aldehyde (II.) with nitro-methane and reduction of the resulting product in the presence of formaldehyde.

1. Formation of diacetylprotocatechuic aldehyde:

(I.) (II.)

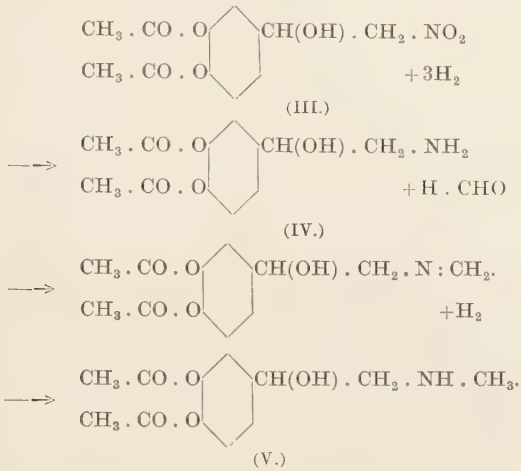
Protocatechuic aldehyde.

2. Condensation of diacetylprotocatechuic aldehyde with nitro-methane:

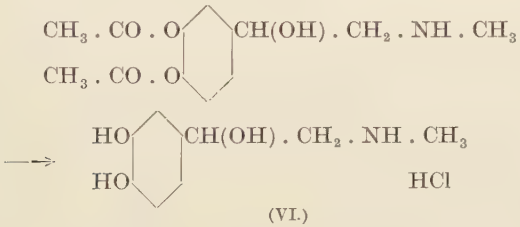


3. The condensation product, diacetyldioxyphenylnitroethanol (III.), is then reduced by zinc and acetic acid in the presence of formaldehyde. The reaction may be explained by assuming that the amino compound (IV.)

is probably first formed, and this reacts with the formaldehyde. The formyl derivative is reduced by the hydrogen produced by the zinc and acetic acid, yielding the methylated amine as follows :



4. Hydrolysis of the diacetyl derivative (V.) with hydrochloric acid yields racemic adrenaline hydrochloride (VI.) :



The details of the method are as follows : A mixture of equimolecular proportions of the diacetylprotocatechuic aldehyde and nitro-methane is shaken together for several hours in the presence of a small quantity of a catalyst at ordinary temperature. Weakly alkaline reagents, such as pyridine, alkali carbonates, etc., are mentioned in this patent specification for this purpose. The condensation product (III.) thus obtained separates

in the form of crystals which are not readily soluble in water or ether, and may be collected by filtration. From the filtrate, by acidification and extraction with ether, some protocatechuic aldehyde may be recovered, the diacetyl derivative being partially decomposed during the reaction. The purified condensation product (III.) is mixed with a 35 per cent. aqueous solution of formaldehyde containing an equimolecular proportion of formaldehyde, and is reduced by acidification with 30 per cent. acetic acid and by the addition of zinc dust, in successive small quantities until four atoms of zinc have been added for each molecule of condensation product. Vigorous agitation is maintained until the crystalline addition compound has almost disappeared. The filtered liquid is then freed from zinc by hydrogen sulphide, and after treatment with hydrochloric acid it is concentrated to small bulk *in vacuo* at a low temperature. Dihydroxyphenylmethylaminoethanol crystallises from the residue after standing in a desiccator.

The Resolution of Racemic Adrenaline

Since the lævo compound is about fifteen times more active physiologically than the dextro modification, the resolution of synthetic adrenaline, which is intermediate between the d- and l- forms in activity, into its optical isomerides, is a matter of great technical importance.

The resolution is accomplished by the fractional crystallisation of the bitartrate from methyl alcohol (Flächer (28), 1908). For this purpose synthetic dl-adrenaline is treated with a little methyl alcohol, and is dissolved in a solution of slightly more than an equimolecular proportion of d-tartaric acid, in hot methyl alcohol. The alcohol is then distilled off *in vacuo* at 35-40° C., and the solution seeded with a crystal of l-adrenaline d-bitartrate (obtained from natural adrenaline). The crystals of bitartrate which separate are quickly dried in a vacuum-desiccator, and are then ground up with a little methyl alcohol, which dissolves out the d-adrenaline-d-bitartrate, leaving the l-adrenaline-d-bitartrate undissolved. The

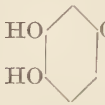
latter is recrystallised from 95 per cent. methyl alcohol or ethyl alcohol (90 per cent.) until the m.p. is 149° C.

From the mother liquors the d-adrenaline is precipitated by ammonia and may be purified by the help of l-tartaric acid. The d-adrenaline may be converted into the racemic base by treatment with mineral acid (D.R.P. 220,355), and from this more l-adrenaline can be obtained by repetition of the above process, until finally all the dl-adrenaline is converted to the active lævo-form.

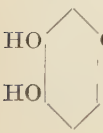
Penicillium glaucum is also stated to effect the separation of d- and l-adrenaline, but the method has no practical application at the present time.

Substitutes for Adrenaline

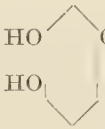
A number of synthetic substances resembling adrenaline in their physiological action have been prepared, but the majority are much less active than adrenaline itself. An exception to this is 3 : 4 dihydroxyphenylethanolamine (*arterenol*)



the pressor action of which is about equal to that of natural adrenaline (Schultz (29), 1909). This substance is prepared by the reduction of aminoacetocatechol or of the cyanhydrin of protocatechuic aldehyde. This synthetic product is of course racemic, and the substance is more active than racemic adrenaline, from which it differs only in the absence of the methyl group as a substituent in the amino group. Attempts to methylate arterenol have not been very successful, however. Other substances which are rather less active are *homorenon* (ω-ethylamino 3 : 4 dihydroxyacetophenone) and *epinine* (3 : 4 dihydroxyphenyl ethyl-methylamine).

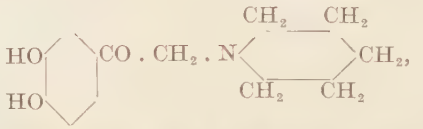


Homorenon.



Epinine.

The latter is said to have about $\frac{1}{8}$ of the activity of natural adrenaline. A quinoline derivative, β-amino α-hydroxy (quinolyl 4) ethane, is claimed to exert a pressor action similar to homorenon, *i.e.* about $\frac{1}{10}$ of the pressor action of l-adrenaline. It is interesting to note that Dakin found that the side chain of adrenaline -hydroxyethylmethylamine—exerts little, if any, pressor effect. *Catechol* is active to a slight extent. Piperidine is known to raise the blood pressure, and Loewi and H. H. Meyer (30) thought that the replacement of the methylamino group of adrenaline by piperidine might produce a substance of very great pressor action. However, piperidoacetocatechol,



prepared in accordance with the above view, was found to be quite inactive. Replacement of the methyl group in adrenalon by a phenyl radicle also leads to the formation of a substance without action on blood pressure, pulse or respiration, although adrenalon itself is weakly active. Alkylation of the two phenolic hydroxyl groups of adrenaline causes the loss of activity of the substance, a fact which shows that these two hydrogen atoms of the catechol ring share in the physiological activity of adrenaline. In general, the adrenaline-like bodies owe their physiological activity chiefly to the presence of an amino group attached to the benzene nucleus by another grouping. The introduction of two hydroxyl groups in the ortho position increases this action, and the presence of a secondary alcohol group between the nucleus and the amino group still more increases the activity of the compound, when these two hydroxyl groups are present.

Physical and Chemical Properties

Dl-adrenaline is a colourless crystalline substance melting at 208° C. — three degrees lower than the

l-modification. According to Takamine it crystallises in five different forms, but it most commonly separates as wart-like clusters of needles. It has a slightly bitter taste, and produces a numbness when applied to the tongue. In the dry form it is stable, and shows a weakly alkaline reaction to moist litmus paper or to phenolphthalein. It is sparingly soluble in cold water (0·0268 per cent. at 20° C.), but slightly more so in hot water. The ordinary organic solvents do not dissolve it, but it is soluble in warm ethyl oxalate and benzaldehyde. Acids or alkalis dissolve adrenaline, the former owing to salt formation due to the methylimino group, the latter on account of the phenolic groups which are present. Adrenaline is precipitated from the solution in acids by the addition of ammonia. The usual alkaloidal precipitants do not throw out adrenaline from solution; but precipitates are formed with ammoniacal solutions of lead or zinc salts. Adrenaline is very readily oxidised by a variety of reagents, especially in alkaline solution, or in the presence of minute traces of ferric salts, with the production of characteristic colour changes (see below). Ammoniacal silver solutions are readily reduced in the cold, and many other metallic solutions, *e.g.* gold chloride, give deposits of the metal. A solution of adrenaline gives an absorption spectrum in the ultra-violet region, which is displaced to the visible region on oxidation (Dhéré) (31).

Natural adrenaline is lævorotatory. The following table shows the specific rotation $(\alpha)_D$ as determined by various observers :

Observer.	Source.	$(\alpha)_D$.	Temp.
Bertrand (1904)	Natural	−53·5°	..
Abderhalden and Guggenheim (1908)	Natural	−50·72°	20° C.
Taveau quoted by Schultz (1909)	Natural	−51·40°	26·4° C.
Flächer (1909)	Resolution of synthetic dl	l −51·40°	19·6° C.
		d +51·88°	19·8° C.
Weidlein (1912)	Whale	−52·00°	25° C.
Abel and Macht (1912)	Bufo agua	−51·30°	20° C.

The melting-point of the natural variety is 211-212° C. (uncorr.) with decomposition. Few crystalline derivatives have been prepared, the majority of the salts being amorphous and deliquescent. The preparation of a stable borate is claimed (D.R.P. 167,317). Pauly has prepared a crystalline urate, and a crystalline bitartrate and oxalate are known, the former melting at 149° C.

A crystalline hydrochloride, m.p. 157° C., is obtained by dissolving a weighed amount of the synthetic base in alcoholic hydrochloric acid, and allowing to remain, with frequent agitation, until the crystals appear (B.P. 1907).

Tests for Adrenaline

Owing to the ease with which colour changes are obtained by the oxidation of adrenaline a very large number of qualitative tests has been described, and no attempt will be made to quote them all, since many only differ in small details.

Vulpian's experiments on the action of iodine have already been referred to. A characteristic pink or rose-red coloration is produced in adrenaline solutions by oxidising agents, of which the following are typical.

1. *Iodine or Iodic Acid.*—In order to increase the sensitiveness of the test the excess of iodine may be removed by shaking with ether (Schur (32)) or by means of sodium thiosulphate (Abelous, Soulie and Toujan (33)). The former method is probably the better, and by this modification the test is said to detect 1 part in 1,500,000.

The use of iodic acid instead of iodine is due to Fränkel and Allers (34) and, independently, Krauss (35). Fränkel and Allers add to the adrenaline solution an equal volume of N/1000 potassium biiodate and a few drops of dilute phosphoric acid. This test is said to be effective at a dilution of 1 : 300,000. According to Bayer (36), 1909, the addition of sulphanilic acid or α-naphthylamine sulphonic acid increases the sensitiveness, intensity and duration of the coloration. This addition is not desirable in conjunction with the simple iodine test since the colour

in this case is similar to that given by other aromatic substances, so that specificity of the original reaction would suffer by this addition ; but with the modified test of Fränkel and Allers the limiting dilution is increased to 1 in five million (Bayer).

2. Another oxidising agent which has been used more recently as a sensitive test for adrenaline is *persulphuric acid* in the form of the sodium or potassium salts. The persulphate solution is added until the concentration in the liquid to be tested is about 1 : 1000, and the contents of the test-tube are then warmed for a short time by immersion in a boiling water-bath (Ewins (37)). The sensitiveness is about equal to that of Bayer's modification of Fränkel and Allers' test, and it is claimed that the red colour produced is not interfered with by the colour of extracts of the gland to the same extent as in the Fränkel-Allers test. According to Borberg (38), 1912, the presence of proteins does not affect this test. In a modification of the reaction suggested by Moreschi (39), 1913, a minute amount of tincture of iodine is carefully floated on the surface of the liquid to be tested. If adrenaline is present a pink colour will be formed at the zone of contact. The colour gradually spreads into the lower liquid and in very dilute solutions disappears rapidly. On then adding a few c.c. of a 1 : 1000 aqueous solution of sodium persulphate a distinct reddish or violet colour will reappear. The test is said to be sensitive to 1 in two millions and the colour persists for several days.

3. Comessatti's reaction depends on the red colour given by solutions of adrenaline on the addition of *mercuric chloride* solutions, and serves to distinguish adrenaline from catechol (Comessatti (40)). The reaction is accelerated by the presence of catalysts ; the metallic salts of weak acids seem to be the most suitable substance for this purpose. Comessatti himself unknowingly supplied this catalyst by using tap-water with which to make up his solution of mercuric chloride : the calcium bicarbonate in the tap-water has been shown to accelerate the reaction. Ewins has suggested the use of sodium acetate for the

same purpose: to 1 c.c. of adrenaline (1 : 100,000) an equal volume of 1 per cent. sodium acetate and then 4 to 5 drops of 1 : 1000 mercuric chloride solution are added. A pale rose tint appears in about five minutes at ordinary temperature, and the test is sensitive to about 1 : 400,000. Recently Stuber, Russman and Proebsting (41) have applied Bayer's modification to this test also. One drop of cold saturated mercuric chloride solution acidified by the addition of 1 per cent. of N 200 sulphuric acid, three drops of cold saturated sulphanilic acid solution, and one drop of N/20 potassium hydrogen iodate solution are added to about 4 c.c. of neutral or feebly acid adrenaline solution. After boiling for one minute, a yellowish brown, reddish yellow or pale yellow coloration is produced which deepens for several hours, and then remains constant. The final tint may be used for colorimetric estimation. The test is claimed to detect 1 : 100 millions.

4. *Ferric chloride* gives the characteristic catechol reaction, namely, the production of a grass-green colour changing to violet and to red on careful addition of dilute alkali. Zechner and Wischo (42), 1921, report the optimum conditions for the simple ferric test as follows: for solutions of adrenaline in concentrations of 1 : 100 and 1 : 1000 and less respectively, one drop of ferric chloride solution of concentration 50, 5 and 0·5 per cent. respectively, to 1 c.c. adrenaline solution are recommended. For more dilute solutions (1 : 1,000,000 and 1 : 10,000,000) 6 to 10 c.c. of solution should be taken for the test, and a control experiment, using water, is desirable in these cases. Bayer has shown that the sensitiveness of this reaction, like that of the iodic acid and chromate reactions, is increased by the addition of sulphanilic acid. With this modification of the test the green colour no longer appears, but a reddish-brown or brown-yellow colour is detectable up to a dilution of about 1 in 300,000.

5. A variety of *other oxidising agents* giving the characteristic red coloration have been described. Potassium ferricyanide (Cevidalli (43)), potassium permanganate, potassium dichromate, and manganese oxides (Zanfrognini

(44)) are among the best known. One drop of 5 per cent. dichromate solution added to 1 c.c. of a 1 : 1000 solution of adrenaline produces a colour range of yellow, orange and red, followed by turbidity and finally brownish flakes (Zechner and Wischo (42)). According to Ogata (45) the brown precipitate is chromium dioxide, CrO_2. Catechol solutions give the same precipitate, and the test is given by other substances containing two adjacent hydroxyl groups. Zanfrognini's reagent is prepared by adding 30 drops of lactic acid to 5 c.c. of 10 per cent. permanganate solution and diluting the solution until it is colourless. This test will detect adrenaline in dilutions of 1 in three millions, and is suitable for the estimation of adrenaline in the suprarenals.

Oxidative ferments also bring about the oxidation of adrenaline (Neuberg (46); Abderhalden and Guggenheim (47)).

The Quantitative Estimation of Adrenaline

Many of the reactions described above have been used for the colorimetric estimation of adrenaline, and in these cases details will be found in the original papers of the respective authors. In the majority of these, however, physiological controls have not been sufficiently thoroughly applied to the methods, and the possibility of errors due to the presence of other phenolic substances must not be lost sight of. Nearly all these methods have been adversely criticised, and at the present time the colorimetric method of Folin, Cannon and Denis (48), 1913, seems to be the only one that compares favourably with the blood-pressure method. Folin, Cannon and Denis's reagent is prepared by dissolving 100 g. of sodium tungstate in 750 c.c. of water, and, after the addition of 80 c.c. of 85 per cent. phosphoric acid, boiling gently for one and a half to two hours. The solution is then made up to 1 litre, and forms an excellent qualitative test for adrenaline (1 in three millions). It also gives an identical colour reaction of very nearly exactly one-third the

intensity with uric acid, and an accurate solution of the latter may be used as a standard for the estimation of adrenaline.

For the assay, the weighed gland is ground with sand and extracted with N hydrochloric acid, by rubbing thoroughly in a mortar. The mixture is then rinsed into a flask, using in all about 15 c.c. of acid for each 2 g. of gland and washing out the mortar with about three times as much water. The acid mixture is then heated to boiling, a little 10 per cent. sodium acetate solution is added (5 c.c. for each 15 c.c. hydrochloric acid), and the boiling is continued. The albumin is promptly precipitated and the whole mixture is transferred (with the exception of the sand) without filtration to a measuring flask (100 c.c. for each 2 g. of gland), and diluted to the mark. The clear specimen for the colorimetric estimation is obtained by centrifugation. For the estimation 5 c.c. of clear extract are measured into a 100 c.c. measuring flask, and 1 c.c. of a fresh uric acid solution (1 mg. uric acid per c.c.) is measured into another. To each flask are added 2 c.c. of the uric acid reagent (see above) and 20 c.c. of saturated sodium carbonate solution. After standing for two to three minutes the solutions are diluted to 100 c.c., shaken and the colours compared in the usual way in the colorimeter, the uric acid standard being set at 20 mm. The adrenaline is calculated as though it were uric acid and the figure obtained divided by three, owing to the three times deeper colour given by adrenaline.

The results obtained by this method agree very closely with those obtained by physiological assay. The method is so delicate that it has been used to show that the blood from the adrenal veins gives a stronger colour reaction after stimulation of the splanchnic nerves than before (Folin, Cannon and Denis, 1913). Kodama (49), 1922, has described a fixed standard for use in the above method, instead of the uric acid solution. The standard consists of 0·01 per cent. water-blue solution (4 c.c.), 0·01 per cent. nigrosine solution (4 c.c.), 10 per cent. $CuSO_4$, $5H_2O$ solution (10 c.c.), 10 c.c. hydrochloric acid (D 1·050) and

water to 100 c.c. This standard, set at 20·6 mm., should match the colour developed by 0·3 mg. uric acid (or 0·1 mg. adrenaline), against which it must be checked.

Johannessohn (50), 1916, finds that Folin's phosphotungstic reagent is not suitable for the estimation of adrenaline when novocaine or alypin is present, and describes a modification of the Fränkel-Allers-Bayer method for use under these circumstances. The estimation of adrenaline in blood has been investigated by Sepulveda (51), 1917. For this purpose the blood serum is added drop by drop to 4 or 5 c.c. of a 5 per cent. solution of mercuric nitrate in N/10 silver nitrate solution until there is complete precipitation. The precipitate is filtered off, and to the filtrate is added drop by drop a standard solution of potassium permanganate until a light rose coloration is produced. Sepulveda finds by this method that normally the adrenaline in the blood reaches a maximum of 0·30 g. per million, and concludes from his results that the determination in pathological conditions has considerable prognostic value.

Kokamoto (52) has used a method depending upon the colour produced by mercuric chloride for the same purpose. He first precipitates proteins from the serum by treatment with absolute alcohol and centrifugation. The addition of mercuric chloride and sodium acetate solutions produces a coloration which will detect fairly accurately the presence of 0·00005 g. of adrenaline in 5·0 c.c. of the serum.

Physiological Actions of Adrenaline

Effects on Blood Pressure.—The most important property of adrenaline lies in its pressor action. As already stated, this fact was first demonstrated by Oliver and Schafer in 1894, when they showed that injections of adrenal extracts raised the blood pressure. Although there are many other naturally occurring substances which possess this property, adrenaline is by far the most powerful. The extraordinary potency of the compound can be judged from the following table, which shows the amount of

adrenaline required to produce an appreciable rise in blood pressure.

Authority.	Animal.	Weight of Adrenaline per Unit of Body Weight.
1. Cameron (53) . . .	Rabbit	0·3 mg. per kilo.
2. Ehrmann (54) . . .	Cat	0·1 mg. per animal.
3. Abel and Macht (55) . .	Dog	0·0013 mg. per kilo.

Magnus (56) demonstrated that adrenaline in dilutions of up to 1 : 20,000,000 was sufficient to produce inhibition of excised plain muscle. It is not surprising, therefore, that minute amounts will affect the blood pressure.

Moore and Purington (57) showed that the injection of very small doses caused a fall in blood pressure. This was explained as being due to the presence of the depressor substance found in all tissue. This explanation seems unlikely, since it is stated that the fall occurs also with the synthetic material. Another striking point is that all authorities (Brown-Séquard (58), etc.) agree that removal of both suprarenals causes death at variable periods, and that a fatal termination cannot be averted by the injection of adrenaline. Whether this is due to the lack of continuous secretion or to some unknown function of the cortex cannot be stated. Indeed the majority of physiologists are coming to the conclusion that adrenaline plays a very unimportant rôle in the maintenance of normal blood pressure (Swale Vincent (59)). The actions of adrenaline can be summed up by saying that they resemble the result of stimulation of the sympathetic, *i.e.* sympathomimetic (Dale and Dixon (60)).

Effects on various Organs.—The results can be summarised in the table on the following page.

Effects on Metabolism

By far the most interesting effects are found upon metabolism. Although large doses may increase the

Organ.	Result.	Authority.
Pupil	No effect unless supr. cervical ganglion destroyed, after which dilatation results. Dilatation in acute pancreatitis	Meltzer and Auer (61). Loewi (62).
Alimentary canal.	Relaxation	Ott (63).
Sphincters pylorus	..	Bunch (64).
Ileocolic internal canal	Contraction	Cannon (65). Elliott (66).
Ureter, urethra, bladder	Relaxation	Swale Vincent (*loc. cit.*).
Genital tract (Uterus, etc.)	Mainly stimulation	Waddle (67).
Bronchioles	Dilatation or constriction, according to strength	Biedl (68). Haplan and Jager (69). Golla and Symes (70).
Arrectores pili	Weak stimulation	Elliott (*loc. cit.*).
Sweat glands	No action	Langley (71).
Salivary glands	Stimulation	Langley (*loc. cit.*).
Lachrymal	..	..
Pancreas	Increases secretion if already secreting. No action on resting gland	Langley (*loc. cit.*).
Stomach	Increased secretion	Yukawa (72).
Respiration	Arrest or decrease	Oliver and Schafer (*loc. cit.*).
Kidney	Volume of urine reduced	Cow (73).
	Solids increased	Addis, Barnett and Shevky (74).
Temperature	Increased	Biedl (*loc. cit.*).

nitrogen excretion in starving animals, little general effect can be found with the exception of the glycosuric reaction.

Blum (75) noted that large doses of adrenaline caused the appearance of sugar in the urine. This worker attempted to explain the picqûre diabetes of Claude Bernard as being due to the same cause. Mayer stated that he was unable to produce picqûre glycosuria after the removal of the suprarenals.

These observations were confirmed by a number of other workers, whilst others, experimenting with dogs, failed to add confirmation. Paton (76) stated that a genuine diabetes mellitus was produced and the syndrome could be induced in an animal rendered glycogen-free by phloridzin. This view has not met with favour, and Achard, Ribot and Binet (77) state that the adrenaline

inhibits glycogen storage. Underhill and Closson (78), however, support a nervous explanation. A careful search through the literature, however, reveals that there is no stable view at the present time.

Adrenaline is said to counteract the effect of insulin. Thus hypoglycæmia can be temporarily relieved by the administration of adrenaline. Glucose must be given in addition, however, if permanent cure is required (McCann, Hannon and Dodd (79)).

Toxic Effects of Adrenaline

The symptoms of an overdose of adrenaline are dyspnœa, excitement, rapid pulse, pyrexia and death (Foà and Pellacani (80)). Epistaxis and hæmaturia may result (Swale Vincent (*loc. cit.*)). The post-mortem signs are indefinite, but the kidneys may show hæmorrhagic changes, and signs of congestion.

Injections of extracts made from suprarenal cortex are said to produce a fall in blood pressure. It has been suggested (Boruttau (81)) that this is due to choline, resulting from splitting off the side chain from adrenaline.

Pearce (82) called attention to the fact that repeated injections of adrenaline caused a marked atheromatous degeneration of the aorta, which was associated with calcification. First described by Josué (83), these observations were confirmed by many other workers (Erb, Fischer, etc.). A very full review of the subject is given in a paper by Saltykow (84), who states that the changes are due to the direct toxic action of adrenaline upon the arteries, and are not the result of high blood pressure.

Distribution of Adrenaline in the Body

The amount of adrenaline present in the suprarenal glands of various animals has been worked out very carefully. The table on the following page gives the results of several workers.

Elliott states that the compound cannot be found in the medulla of the fœtus, but that it is present in some of the other chromaffin tissues, such as the paraganglion

Authority.	Animal.	Percentage Wet.	Percentage Dry.
Abel (*loc. cit.*) . .	Ox	0·3	1·8
Hunt (85) . . .	Ox	..	1·5
Abel (*loc. cit.*) . .	Sheep	0·25	..
..	Calf	0·34	..
..	Cattle	0·39	..
Herring (86) . .	Rat	0·073 mg. per 100 g. body weight.	
..	Rabbit	0·083 g. per kilo.	
Ornstein (87) . .	Guinea-pig	0·229 g. per kilo.	
Manufacturer's yield : 0·095 to 0·103 per cent.			
Elliott (88). . .	Human	0·1 per cent. total 8-9 mg.	
Isigier and Schnol (89):			total 4·6 mg.

aorticum. Fevers, septic conditions and pneumonia cause a lowering in the content, whilst normal figures were obtained from cases dying from chronic nephritis and high blood pressure.

Shigenoku Kuriyama (90) found that the adrenaline content of aseptic glands decreased markedly on keeping. That this was due to oxidation was proved by the absence of such changes in an oxygen-free medium. Starvation does not affect the amount in the glands, nor do daily injections or intoxication with adrenaline increase the quantity. The amount can be reduced by the injection of diphtheria toxin. Ohno (91), using Ogata's silver method, found that the adrenaline content of the suprarenals was markedly increased in beri-beri. An average of five cases gave 9·45 mg. as the total quantity present in both glands. McCarrison (92) also noted that in the deficiency œdemas resulting from feeding pigeons with autoclaved rice, the adrenaline content of the glands was markedly increased. Since the condition can be relieved by butter, he suggested that this food might contain some substance which was responsible for keeping the adrenaline content within normal limits.

One of the most extraordinary occurrences of adrenaline was found by Abel and Macht (93), who noted the presence of the base in the saliva or venom of the tropical toad *Bufo agua*. As much as 5 per cent. may be found in the dried secretion.

Mode of Synthesis of Adrenaline in the Body

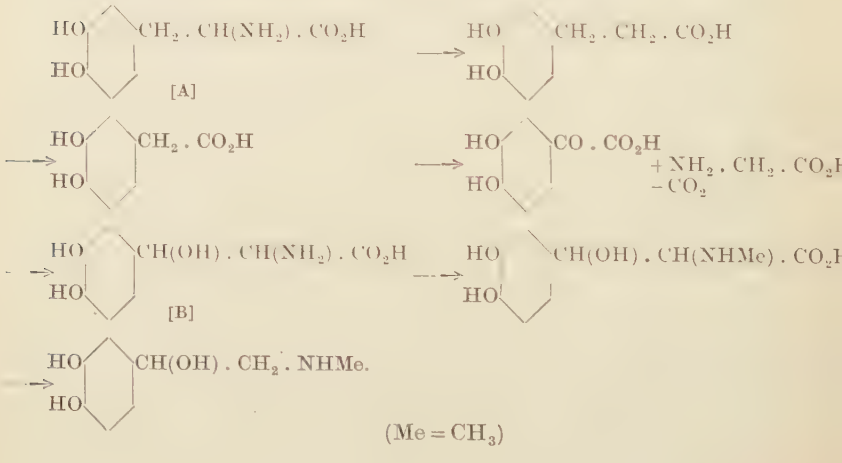
Nothing is known about the precursors of adrenaline. Many theories have been advanced, but they have found little experimental confirmation.

Halle (94), 1906, maintained that adrenaline increased in quantity when the glands were incubated with tyrosine. Ewins and Laidlaw (95) failed to confirm this observation. Still earlier Abelous, Soulie and Toujan (*loc. cit.*) stated that adrenaline resulted from the incubation of the gland with muscle. von Fürth (96), in his article in *Abderhalden's Biochemisches Handlexikon*, says that the idea of the formation of adrenaline from tyrosine and tryptophane in autolysis should receive no support. Friedmann (97) put forward the idea that adrenaline could be formed by the methylation of oxyphenylserin, followed by decarboxylation :

$(OH)C_6H_4 . CH(OH) . CH(NH_2) . COOH$
$\rightarrow (OH)_2C_6H_3 . CH(OH) . CH(NH . CH_3) . COOH$
$\rightarrow (OH)_2C_6H_3 . CH(OH) . CH_2 . NH . CH_3.$

There is no support for this theory.

Rosenmund and Dornsaft (98) were led to suggest the following possible synthesis of adrenaline through some work on the preparation of phenyl serins from phenyloxyacrylic esters by the addition of ammonia.



They state that possibly [B] is a constituent of proteins, but that it is either present in exceedingly small quantities or that it is destroyed in hydrolysis, and consequently is unknown. This view as to a possible synthesis in the body has been vigorously attacked by Knoop (99), who states on theoretical grounds that the reactions are most unlikely to occur.

The Interaction between Adrenaline and other Substances

It has already been stated that adrenaline counteracts the symptoms of hypoglycæmia. Lyman, Nicholls and McCann (100) demonstrated that the blood-sugar curve following the injection of insulin could be greatly modified by adrenaline. McCann, Hannon and Dodd (79) stated that the inhibition was of a temporary nature, and that injections of adrenaline in hypoglycæmia were not to be recommended.

Stewart and Rogoff (101) studied the effect of drugs upon the output of adrenaline. Strychnine, in doses so small that no reflex exaggeration occurred, caused a prolonged output of adrenaline. Moreover, they could detect no decrease in the adrenaline content of the suprarenals, and they suggest that the action takes place rather through the nerves than by a direct action upon the glands. The intravenous injection of concentrated salt solutions (sodium carbonate) produced the same results. Nicotine and curare diminish the production of adrenaline.

Elliott's Method for the Estimation of Adrenaline

If it is desired to estimate the concentration of adrenaline in glands, they are dissected and washed. The tissue is then cut up into small pieces with scissors, and ground up in a mortar with a little sand and Ringer's solution. The paste is washed out into a glass vessel with more Ringer's solution, and the total volume is made up to 15 c.c. The mixture is brought rapidly to the boil, and is filtered through glass wool. The resulting filtrate is employed for injection. Elliott considers that the error

due to the fact that the solid in the solution is not allowed for is balanced by the loss by evaporation during the boiling.

In the meantime, a cat is anaesthetised, and the brain is destroyed by pushing a probe upwards through the foramen magnum. Artificial respiration is then carried on by inserting a tracheal cannula after cutting both vagi. A blood-pressure cannula and apparatus are now fitted up. At this stage the pressure is high, 140 mm. Hg. or so. The cord is next destroyed down to the level of the 4th thoracic segment by inserting a long probe through the orbit.

The blood pressure immediately falls to about 40 mm. Hg., and, after a short time, it remains constant at this level.

The conditions will now be suitable for quantitative experiments. Injections can be made into the external jugular vein by means of a cannula connected with a burette containing Ringer's solution.

A standard solution of adrenaline is first injected. Elliott employs 1 c.c. of Höchst's synthetic suprarenin (0·1 per cent. synthetic diluted to 0·0025 per cent.). The volume, 1 c.c., must be injected in five seconds, as judged by a metronome. This gives a typical curve, as recorded on the paper of the kymograph. When the pressure has returned to normal, 1 c.c. of the unknown extract can be injected under the same conditions. The potency of the preparation can be calculated by comparing the rise in blood pressure with that given by the standard quantity. By this means Elliott claims that the base can be assayed to an accuracy of 0·01 mg.

The Preparation of Adrenaline Solution for Clinical Use

Adrenaline is usually dissolved for clinical use in a dilution of 1 in 1000. A preservative is usually added, and the solution is made isotonic. Sometimes sulphur dioxide is dissolved in the solution to inhibit oxidation

and corresponding loss of activity, and some commercial preparations contain sulphites. The solution is usually stored in ampoules filled with great care in an atmosphere of carbon dioxide. Sterilisation by heat is not recommended by Richard and Malmy (102), but Wischo and Zechner (*loc. cit.*) oppose this view.

Finnemore (103), 1907, suggests the following preparation : Adrenaline 0·10, chlorbutyl alcohol 0·50, sodium chloride 0·90, dilute hydrochloric acid 0·25, sulphurous acid 0·25, distilled water to make 100 parts. Richard and Malmy (*loc. cit.*) urge the adoption of a standard 1 : 1000 solution. They find that solutions containing sulphur dioxide are best, the SO_2 acting as solvent, antiseptic and preservative. According to their method the adrenaline (1 g.) is dissolved in 100 c.c. of physiological salt solution (0·75 per cent.) containing 10 g. of sulphur dioxide per litre (controlled by iodometric titration with N/10 iodine solution), and the solution is made up to 1 litre with previously sterilised 0·75 per cent. sodium chloride solution. The solution must be made in the absence of fumes of ammonia, which quickly colour it. Such solutions have been preserved for a year without any loss of activity.

Debucquet (104) dissolves the base in cold saturated benzoic acid solution containing 7 g. of sodium chloride per litre.

Chemical Incompatibilities of Adrenaline

Many iron and arsenic preparations have the property of destroying the action of adrenaline on the blood pressure. According to Valeri (105) the following preparations act in increasing intensity in the order named : sodium arsenite, iron lactate, iron citrate, iron sulphate, iron albuminate and ferratin. The experiments were controlled by observations on the blood pressure of dogs and the iris of frogs. Adrenaline is also incompatible with iodine and with mercuric chloride. Piccinini (106) states that pure adrenaline solutions lose their activity

completely in thirty days in the presence of ferrous salts or arsenates, whereas the complete suprarenal extract retains its activity under the same conditions.

Nomenclature

Few substances have received such a variety of names as the active principle of the suprarenal gland, and there is still a complete lack of uniformity in the nomenclature adopted. The principal names commonly in use to-day are adrenalin or adrenaline (Takamine), suprarenin (v. Fürth), epinephrin (Abel) and adrenine. The one in most general use in this country is adrenaline, and it is also the name given to the substance by Takamine, who was the first investigator to obtain the active principle in the pure state. The objection has been raised that "adrenalin" is a trade-name given by certain manufacturers to their products. Nevertheless, the above reasons have led to the use of the name "adrenaline" in this book. The hydrate theory of Abel, already referred to, and the fact that the name epinephrin was applied by him to a substance which was certainly not identical with adrenaline, make the adoption of this title for the active substance somewhat misleading. The only objection to the names suprarenin and adrenine is that they are not those commonly employed for the substance, although the latter has now been adopted by the *Journal of Physiology*.

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CHAPTER VI

MISCELLANEA

In this final chapter some of the more obscure internal secretions are dealt with. After careful consideration it has been decided to include only those upon which a fair number of observers have worked, and to exclude such controversial subjects as the secretions of the pineal, thymus and cortex of the suprarenal. Secretin, parathyroid preparations and spermine will be dealt with fully, since there is a large body of work upon these subjects. Spermine was only included after some hesitation, since the physiological effects of this compound have not been carefully worked out. It was thought advisable to include it, since any one about to undertake investigations upon this point would necessarily have to become acquainted with the literature dealing with this body.

Secretin

Methods of Preparation.—In 1902 Bayliss and Starling (1) showed that the injection of extracts of the duodenal mucosa caused a copious secretion from the pancreas, even after its nervous connections had been severed. The active principle of these extracts was named secretin. Their method of preparation consisted in scraping off the mucosa of the fresh duodenum and jejunum, and grinding up the shreds with sand and dilute hydrochloric acid. When a uniform paste had been produced, the mixture was carefully neutralised and filtered. Intravenous injection of this solution caused the pancreas to secrete

vigorously. Ten years later Dale and Laidlaw (2) modified this process very considerably, and were able to prepare a highly active solution. Their method is as follows: the upper two-thirds of the small intestine of freshly killed dogs are opened, and the moist mucosa is scraped off and is weighed. This material is then thoroughly ground up with one-fifth of its weight of solid mercuric chloride until a smooth, uniform paste is obtained. Two c.c. of water are added for each g. of mucosa, and after stirring, the mixture is set aside until sufficient material is collected to work up a large batch. The whole is boiled and filtered, the filtrate being rejected. The solid matter is pressed dry, and is then broken up into small pieces, which are suspended in a solution consisting of 2 per cent. acetic acid and 1 per cent. mercuric chloride. Dale and Laidlaw recommend the use of 4 c.c. of this mixture for each gram of membrane taken. The mixture is then brought to the boil and filtered. Ten per cent. sodium hydroxide is then added to the clear filtrate until the reaction approaches that of neutrality. This can be judged by the fact that the yellow precipitate of mercuric oxide will just not remain permanent. On standing, a white flocculent precipitate appears, which is collected either by centrifugation or filtration. This precipitate consists of a mercury compound of secretin. It is suspended in water, and freed from mercury by the passage of hydrogen sulphide. The insoluble mercuric sulphide is filtered off, and the solution is neutralised. After boiling off the sulphuretted hydrogen, a strongly active solution is obtained.

The addition of picric acid will precipitate the active principle. Alternatively the white precipitate may be decomposed by treating with hydrogen sulphide in the presence of 75 per cent. alcohol. After neutralisation and filtration the secretin can be precipitated by the addition of an excess of acetone.

Practically all the possible permutations and combinations of these two methods have been employed by various workers. Thus von Fürth and Schwartz (3) extracted

the mucosa with dilute acetic acid in place of hydrochloric acid. Frouin and Lalou (4) showed that the amount of secretin produced was roughly in proportion to the hydrogen ion concentration of the extracting medium, and was therefore independent of any particular acid radicle. Stepp (5), 1912, repeated much of the earlier work, and showed, in addition, that secretin could be extracted by means of 70 per cent. alcohol, or strong soap solutions. Matsuo (6) stated that a 0·6 per cent. solution of sodium chloride was equally efficient as acid or alcoholic extractants. Beveridge (7) preferred to heat the mucosa with 0·1 to 0·8 per cent. hydrochloric acid for some time just below boiling-point. Dalman (8) claims that a highly potent secretin of low toxicity could be prepared by precipitating secretin solutions by the addition of 9 volumes of acetone.

It has been suggested that secretin is present in the mucosa as an inactive parent substance –prosecretin, and that all methods of preparations depend in the first place upon breaking down this substance to the active principle. Lalou (9), 1913, produced evidence to the contrary, and maintains that free secretin is present in the mucosa. This would be supported by the fact that the body can be obtained by extraction with inert solutions, such as 0·6 per cent. saline. Stepp and Schlagentweit (10) confirmed Lalou's views.

Various attempts have been made to extract secretin from sources other than those mentioned. Bickel (11) recorded the preparation of both gastric and pancreatic stimulants from spinach. The compounds are very firmly bound, and prolonged boiling with water, or hydrolysis with hydrochloric acid, is required for their liberation. In a later paper (12) he showed that the principles were more active when administered intravenously than when given by the mouth. Eisenhardt (13) confirmed these observations, and stated that the active substance was contained in the arginine-histidine fraction. In all three of these papers probable associations of secretin with vitamins is discussed.

Van Eweyk and Tennenbaum (14) state that protein hydrolysates have no secretin like properties. Later, Van Eweyk, working with Bickel (15), states that if material obtained from the hydrolysis of casein be heated to 155 to 165° C. it assumes secretin-like properties. They also state that if dried egg yolk is heated to 130° C., and subsequently hydrolysed, the resulting product is inactive unless heated, as in the case of casein. They suggest the term "heat-secretin" for these bodies.

Van Eweyk and Tennenbaum (16) in 1921 recorded the preparation of a secretin-like substance from spinach. This body had a marked action upon gastric secretion, but had no depressor action.

Edkins (17) found that extracts of pyloric mucosa, when injected, caused a stimulation of gastric secretion. This body has been called gastric secretin, in contradistinction to pancreatic secretin.

Properties.—Nothing is known of the constitution of secretin other than that it is of a protein-like nature. Various suggestions as to its probable constitution have been made. Thus, since histamine causes a flow of pancreatic juice, it has been stated that this substance may be responsible for some of its actions. That this is not so can be readily proved by the absence of any marked action upon the uterus. Popielski (18) maintains that the effect of secretin is due to vasodilatation, and is non-specific. Bayliss and Starling (*loc. cit.*), however, were able to avoid the depressor effect of secretin by boiling the mucosa with absolute alcohol prior to treatment with acid. The work of Dale and Laidlaw (*loc. cit.*) seemed to show that although the depressor action of secretin might be due to histamine, the stimulatory powers were not. On the other hand, it would appear that secretin is not a very complex body, since it diffuses through parchment and does not give rise to antibodies. Lalou found that its activity was rapidly destroyed by gastric and pancreatic juices, and also by erepsin.

Distribution.—Secretin is best prepared from the upper two-thirds of the small intestine, although it may be

present at lower levels. The majority of observers state that it cannot be obtained from other tissues, although Popielski (*loc. cit.*) holds the view that it is a non-specific vasodilator. Pringle (19) stated that secretin was present in the intestines of new-born animals, but that it was not invariably found in the alimentary canal of embryos.

Physiological Actions.—Since the physiological properties of secretin involve a discussion of the whole of the various theories concerning alimentary secretion, only a very brief summary will be given here. It will be remembered that Bayliss and Starling (*loc. cit.*) showed that the passage of acid into the duodenum causes a liberation of secretin into the blood stream. After circulating, it reaches the pancreas *via* the blood stream, and stimulates secretion.

The presence of various substances such as acid, soaps, urea, etc., in the duodenum is capable of giving rise to the production of secretin. These changes can be induced by the intravenous injection of secretin, even after the nervous connections of the pancreas have been severed.

The *chemistry* of the juice secreted under the influence of secretin has received little attention. Bayliss and Starling state that it is similar in every respect to that secreted under normal stimuli. John Mellanby (20), however, in a recent preliminary communication, showed that, whereas the alkalinity of the juice was unaffected, the enzyme content of secretin juice fell steadily. Secretin preparations are all depressor in action; the extent of the fall depending upon the purity of the material.

As already indicated, it has been suggested that secretin and the vitamins are allied. Some have even gone so far as to state that secretin and vitamin B are identical. Cowgill (21), and Anrep and Drummond (22) have proved that this is not so. Downs and Eddy (23) showed that secretin improves the blood picture of anæmic rabbits, but they state definitely that they do not consider that this is any support to the theory that secretin and vitamin B are allied.

In conclusion, a brief reference may be made to the

clinical application of secretin. This has been advocated by many workers in diabetes mellitus, since in these cases a diminished amount of secretin has been found. There is no evidence, however, that secretin has any therapeutic virtues. The whole question is reviewed very fully by Carlson, who states that, since secretin is inactive unless administered intravenously, its clinical applications are nil, owing to the danger of injecting such an impure substance.

The Parathyroid Glands

Historical.—The intimate anatomical relationship between the parathyroid and the thyroid glands is probably responsible for the comparatively late date at which the parathyroids were discovered. The two external glands were first described by Sandström (24) in 1880, and the two internal ones by Kohn (25) fifteen years later. At first these glands were thought to be accessory thyroids and to fulfil the same functions. Thus Gley (26) pointed out the high iodine content of these glands, and brought forward other arguments which, he claimed, showed their close association with the thyroid. Moussu (27), however, took the contrary view and emphasised the dissimilarity in function and structure of the thyroid and parathyroid glands. These two views are still held, and it cannot be definitely stated that there is at present any generally accepted opinion on the matter.

During the next twenty years clinical observations led to the association of the function of these glands with calcium metabolism. Koch (28) in 1913 showed that an excess of guanidine derivatives was present in the urine of parathyroidectomised dogs, and Noël Paton (29) and others found that the tetany following parathyroidectomy was accompanied by an increase in the quantity of guanidine in the blood.

The application of parathyroid-therapy has in view these two objects: (1) the control of calcium metabolism, and (2) the rendering harmless of certain toxic substances such as guanidine and its derivatives.

The Chemistry of the Parathyroid.—Very little is known about this subject. Vassale (30) prepared a substance which he named "parathyroidin", but details of the process are not given. Berkeley and Beebe (31), from the delicate nature of the compound, considered that the active principle is an enzyme. They state that when the proteins are extracted by normal saline from the parathyroids, the active fraction is contained in the precipitate obtained by addition of acetic acid ("nucleoprotein"). The presence of colloid material in the parathyroid has been shown by a number of workers, but the general view is that this plays no part in the activity of the gland.

There is so much difference of opinion as to the *iodine content* of the parathyroid, that it is only possible to record some of the very varied results obtained. It is probable that, as Estes and Cecil (33) suggest, the difficulty of obtaining parathyroid tissue quite free from thyroid is to some extent responsible for this. Gley (3) stated that the parathyroid contained proportionately thirty-five times as much iodine as the thyroid. Berkeley and Beebe were unable to demonstrate the presence of iodine. Chenu and Morel (32) showed that by using larger quantities of the glands the presence of iodine could be demonstrated. Thus the tissue from eight dogs (0·133 g.) gave an iodine content corresponding to 0·0563 mg. of iodine per gram of gland. Estes and Cecil found only traces of iodine.

Vines (34) has found that preparations of the parathyroid act upon guanidine *in vitro* under certain conditions, and has based a method of standardisation upon this observation. The guanidine is estimated in the test-fluid before and after the action of the preparation. The details of the method are as follows: approximately 1 g. of guanidine carbonate is dissolved in distilled water, the solution is made just acid to phenol red with acetic acid, and the volume is made up to 100 c.c. For each test, 10 c.c. of this solution are used. To it are added 20 c.c. of 0·85 per cent. sodium chloride solution, 1 c.c. of 1 per cent. calcium chloride solution, and a small quantity of

thymol: this constitutes the test-fluid, and the amount of guanidine in it is estimated as the picrate. To test the activity of the preparation 0·065 g. is added to the test-fluid, and the mixture is incubated at 37° for four days with continual aeration by suction. At the end of this time the guanidine is once more determined as picrate, and the difference in milligrams between this figure and that of the original test-fluid is Vine's arbitrary index of activity.

The following table, reproduced from Vine's book (34), shows the actual figures obtained from a series of commercial preparations:

Manufactured by	Activity in Arbitrary Units.		
	Desiccated Powder.	Tablets.	
A	100, 93, 100	60, 82, 68, 84 68, 76, 69, 66 74, 59, 63, 74	Group I.
C	86	..	
G	..	67	
I	..	60	
A	67	..	Group II.
B	66	..	
C	66	..	
F	56, 50	..	Group III.
E	38	..	
H	..	31	

The chemistry of this reaction has not yet been worked out. There is, however, no evidence for the most obvious chemical change, namely, the conversion of guanidine to urea.

In the *clinical application* the desiccated gland is given by the mouth in daily doses of one-tenth of a grain. This dose can be continued for comparatively long periods without ill effects.

Many rigid controls, however, must be performed before these views upon the parathyroid can be considered as proved.

Spermine

Historical.—The occurrence of a peculiar crystalline body in certain parts of the animal organism, and particularly in semen, has been the subject of study for the last two and a half centuries. This substance, characterised by its occurrence in "glittering, translucent crystals", was first described by Leeuwenhoek (35) in 1678, and Vauquelin (36) a century later rediscovered the compound, being evidently unaware of Leeuwenhoek's observations. From the middle of the nineteenth century a series of papers appeared by Charcot and Robin (37), and other workers, who assigned very different constitutions to the substance, the crystals of which appear to be plentiful in patients suffering from bronchial asthma or from leucocythæmia. Thus Friedrich (38) had supposed that they were crystals of tyrosine; Harting (39) believed them to be calcium phosphate; Böttcher (40) expressed the view that they were albuminoid bodies, and Forster (41) and Salkowski (42) regarded them as mucous compounds.

Chemistry.—The first really systematic chemical investigations on this subject are due to Schreiner (43), who in 1878 isolated and purified the substance, showed that it is an organic base, and prepared and analysed a series of crystalline derivatives. According to Schreiner the crystalline substance occurring in the organism is the phosphate of this base. The crystals form 5·237 per cent. of the solid constituents of human semen, and can be easily obtained by boiling the fresh fluid with alcohol. The precipitate is dried at 100° C., extracted with warm water containing a few drops of concentrated ammonia, and the alkaline solution is evaporated, when the crystals appear. The crystals also separate out on the surface of pathological preparations which are preserved in alcohol.

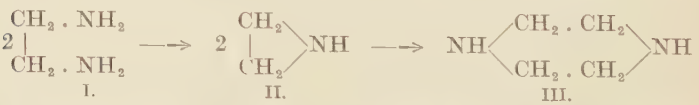
After purification by recrystallisation from hot water containing a small quantity of ammonia, the crystals, which are in the form of prisms and double pyramids, are colourless and brittle. They are insoluble in ether,

chloroform, alcohol and in cold water, but dissolve readily in dilute acids or in solutions of caustic alkalies or alkali carbonates.

The salt which occurs in the organism contains two atoms of nitrogen to one of phosphorus; it loses three molecules of water at 100° C., melts at 170° C. and decomposes at a higher temperature with the evolution of ammonia. By precipitation of the phosphoric acid by means of baryta, Schreiner obtained the free base as a colourless odourless crystalline mass, having a bitter taste. It was soluble in alcohol, insoluble in ether, and its solution immediately formed a crystalline compound on the addition of phosphoric acid.

Schreiner purified the base by adding lead acetate to the solution of the crude phosphate, removing excess of lead from the filtrate by hydrogen sulphide, and precipitating the base from the filtrate by phosphotungstic acid. The free base, obtained by boiling the phosphotungstate with baryta, formed a crystalline hydrochloride, to which Schreiner assigned the formula, C_2H_5N . HCl. He also prepared the platinichloride and the aurichloride (C_2H_5N . HCl, $AuCl_3$), the latter crystallising in golden plates, soluble in ether, alcohol and water.

These analyses pointed to the structure of the base as belonging to a class of compounds unknown at the time of Schreiner's work. It was not until ten years later that Ladenburg and Abel (44) attempted to prepare ethylene-imine (II.) from ethylenediamine (I.) by dry distillation of the hydrochloride, and showed that there is strong evidence to suppose that ethylene-imine was first formed in the distillation and quickly became converted to the dimeric form piperazine (III.), as was shown



by determination of the vapour-density. Ladenburg and Abel prepared numerous crystalline derivatives of ethylene-imine, and considered that there was strong

reason to suppose that ethylene-imine was identical with the substance spermine, obtained by Schreiner.

Majert and Schmidt (45) in 1890 wrongly concluded from a series of experiments on the comparison of diethylenediamine (first obtained by Hofmann), spermine (Schreiner), ethylene-imine and piperazine (Ladenburg), that all these substances were identical, and suggested that Ladenburg was dealing with the carbonate of the base, which is readily formed on exposure to the carbon-dioxide of the air.

In the following year the same authors (46) carried out direct comparison of spermine and piperazine and found that, although there was a great similarity between the two substances and some of their salts, they are not identical. In particular, the bismuthoiodides and the phosphates showed marked differences.

Poehl (47) has independently confirmed this view. The properties of the base agree fully with those described by Schreiner. The analysis of the platinichloride, however, gave numbers which did not agree with Schreiner's empirical formula C_2H_5N, but with the formula $C_{10}H_{26}N_4$, and the analysis of the aurichloride confirmed this formula. Spermine can therefore be neither identical nor isomeric with piperazine. This view has been fully confirmed by recent work.

Schreiner (*loc. cit.*) has pointed out that spermine is not specific to human semen, but is widely distributed throughout the animal organism.

Owing to the incompleteness of experimental detail which is found in Schreiner's original paper, this result was generally doubted until it was recently confirmed by Rosenheim (48), who prepared the substance from the tissues of both male and female animals, and, in addition, from yeast.

The most striking property of spermine is the insolubility of its phosphate. This, combined with its marked tendency to crystallise in a very characteristic form, led to the early discovery. The yields obtained from various tissues are very small, and vary from about 0·003 to

0·025 per cent., the latter quantity being obtained from the pancreas. In two recent communications the whole subject is considered very carefully (O. Rosenheim (49); Dudley, Rosenheim, M. C., and Rosenheim, O. (50)), the latter paper being the first part of a series upon the chemical constitution of spermine. A very full selection of references to the history of the subject will be found at the end of the former paper. The following descriptions are mainly due to these two communications.

Preparation of Spermine Phosphate from Pancreas

Of the three methods of preparation described by Dudley, Rosenheim and Rosenheim (*loc. cit.*), the simplest appears to be the following: 5 kg. of minced fresh pancreas (ox) are mixed with 10 litres of boiling water, and the mixture is brought to the boil; 30 c.c. of glacial acetic acid are then added. After cooling, the mixture is filtered, and the filtrate is evaporated *in vacuo* to as small a bulk as possible. Solid sodium hydroxide (50 g. per 100 c.c. fluid) is added, and the solution is subjected to distillation in steam until the distillate no longer gives a precipitate with Dragendorff's reagent. This indicates that all the spermine has been distilled over. The volume of the distillate is accurately measured. One-third is withdrawn and is titrated with 10 per cent. phosphoric acid, using methyl orange as an indicator, and finally adding sufficient phosphoric acid until the reaction is distinctly acid to sensitive Congo paper. The remaining two-thirds of the basic solution are then added, and the solution is evaporated to about 500 c.c. Spermine phosphate crystallises on the addition of sufficient alcohol to produce a concentration of 25 per cent. The yield is about 1·5 g. from 5 kg. of pancreas.

Spermine phosphate after recrystallisation from water is obtained as colourless crystals, insoluble in alcohol, ether and organic solvents, easily soluble in dilute acids and alkalies. One part of the salt dissolves in 100 parts of boiling water. At 20° C. the solubility is 0·037 per cent.

The melting-point is 230-234° C. (softens 227° C.) with evolution of gas. The analysis of specimens from various sources agrees with the formula $C_{10}H_{26}N_4 . 2H_3PO_4 . 6H_2O$.

The *picrate*, $C_{10}H_{26}N_4 . 4C_6H_3O_7N_3$, on heating blackens at 242° C. and melts sharply at 248-250° C. It is sparingly soluble, and may be recrystallised from a large volume (200 parts) of boiling water, when it separates in slender yellow needles.

Other salts, such as the gold and platinum salts, have been fully investigated by Dudley, Rosenheim and Rosenheim. The *chloroaurate*, $C_{10}H_{26}N_4 . 4HCl . 4AuCl_3 . H_2O$, melts at 225° C.

The *chloroplatinate*, $C_{10}H_{26}N_4 . 2H_2PtCl_6$, m.p. 242-245° C., crystallises very well, and the crystalline form has been shown to be the same in the case of specimens derived from semen and from pancreas (T. V. Barker, quoted by O. Rosenheim (49)).

Tetra-benzoyl and phenylisocyanate derivatives have also been prepared. The molecular weight determinations of the former indicate a molecular weight corresponding with the formula $C_{10}H_{26}N_4$ for spermine.

Spermine itself is obtained by adding 50 per cent. potassium hydroxide to a concentrated solution of the hydrochloride or a suspension of the phosphate in water. The free base is extracted with chloroform. It is obtained as a colourless oil, which rapidly solidifies to a mass of needle-shaped crystals melting at 55-60° C. It may be distilled *in vacuo* without decomposition, and boils at 150° C. at 5 mm. It is easily soluble in water, ethyl or butyl alcohols, and is insoluble in benzene, ether or ligroin.

The base readily absorbs carbon-dioxide from the air and liquefies. It is optically inactive.

With regard to the physiological activity of spermine nothing is known. The value of commercial preparations containing the base has yet to be demonstrated.

Addendum to Chapter VI

A very important series of observations on the hormone of the parathyroid glands has appeared since the above section was written. These are contained in three recent papers of Collip and his co-workers (Collip, J. B., *Amer. J. Physiol.*, 1925. lxxii. *Proc. Amer. Physiol. Soc.* 182; Collip, J. B., *J. Biol. Chem.*, 1925. lxiii. 395; Collip, J. B., Clark, E. P., and Scott, J. W., *ibid.* p. 439).

In these experiments the glands were collected from freshly killed oxen, and after dissection were frozen at once and stored at -4° C. When required for use, a few glands were placed in a large test-tube, covered with an equal volume of 5 per cent. hydrochloric acid, and were heated in a boiling water-bath for one hour. The layer of fat was then removed and the cooled liquid made alkaline to pH 8 by the addition of sodium hydroxide. Hydrochloric acid was then added slowly until the maximum precipitation occurred. This precipitate was collected, either by centrifugation or by filtration, and was redissolved in weak alkali and again precipitated. The supernatant liquors (or filtrates) are combined, and these consist of an aqueous extract of the active principle.

This solution can be administered either (1) by stomach, (2) by subcutaneous injection, or (3) intravenously. The extract can be sterilised for (2) or (3) by heating in a boiling water-bath. Collip states that the hormone appears to be a fairly simple organic substance. The extract is crystal-clear and causes no local reaction. The active principle is insoluble in ether, but it is soluble in alcohol, at least up to 92 per cent. concentration. Desiccated glands have not given satisfactory results in Collip's hands.

The physiological action of the extracts has been tested upon dogs, using both normal animals and those after complete thyroparathyroidectomy. In the latter case, dogs usually developed fatal tetany in twenty-four to forty-eight hours, but where doses of the hormone were

given once or twice daily, tetany did not result, even when a heavy meat diet was given. The blood calcium was increased, the maximum effect being a few hours after injection. Death from hypercalcæmia resulted from an overdose of the hormone. The symptoms of hypercalcæmia are vomiting, diarrhœa, and general atonia, and the circulation becomes impaired.

With normal dogs the effect of changes in (1) the size of dose and (2) the time between injections has been studied. The results indicate that (1) is of little importance, while (2) is of the greatest significance. Thus, by successive injections each twenty minutes for eight hours, values of the blood-calcium up to 21·5 mg. Ca per 100 c.c. have been obtained. The following is a summary of the results : *Viscosity*, great increase ; *osmotic pressure*, + 10 to 15 per cent. ; *phosphates*, + 100 per cent. ; *non-protein nitrogen* up to 400 per cent. increase. The *urea nitrogen* shows a similar increase to the non-protein nitrogen ; the total *protein-content* of the blood is increased ; whilst the *blood-halogen* decreases by 10 to 15 per cent. The *blood volume* is diminished by 5 to 15 per cent., and the *alkali-reserve* also shows a diminution. The *blood-sugar* is little affected. Collip concludes that the extract is effective either by the oral route or by injection, although preferably given by the latter method, and affords a complete replacement therapy.

The extract has been administered by injection into a number of human subjects, causing an increase in the blood-calcium. Excellent results were obtained in one case of infantile tetany, and also in a case of acute nephritis with extensive œdema in a child of five. This branch of the application will no doubt be further developed, but in any case there seems to be little room for doubt that Collip has succeeded in obtaining a very potent extract which contains the active principle of the parathyroid glands.

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